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- **Clinical applications and recent advances in radiotherapy for the management of neoplasia in companion animals** (P: 1-9)
- **A Comprehensive Review of Microneedling and Facial Skincare Strategies in Anti-Aging Dermatology** (P: 11-30)
- **ABCB1 Promoter Methylation: A Potential Biomarker and Therapeutic Target in the Pathogenesis of Ulcerative Colitis** (P: 31-37)
- **Pathological aspects of Trombidiosis in Crested lark (*Galerida cristata*)** (P: 39-41)
- **The protective effect of thiamine on the histo-stereological structural of the testicles and oxidative state induced by copper oxide nanoparticles in rat** (P: 43-51)
- **Hamburger standard in Iran: Mini review** (P: 53-46)



ORIGINAL RESEARCH PAPER

Clinical Applications and Recent Advances in Radiotherapy for the Management of Neoplasia in Companion Animals

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Abstract

Cancer is among the most common causes of illness and death in companion animals, creating a strong need for effective and accessible treatment strategies. Radiation therapy (RT) has become a cornerstone in veterinary oncology, applied as an adjuvant following incomplete surgery, as a curative option for tumors not amenable to resection, and as palliative care to reduce pain or dysfunction while improving quality of life. Recent advances in treatment planning and delivery have significantly improved the safety and precision of RT. Modern imaging, computer-assisted planning, and image-guided techniques now allow more accurate targeting of tumors while sparing surrounding normal tissues. Alongside conventional external-beam therapy, selective use of brachytherapy, plesiotherapy, and radionuclide-based approaches—such as radioiodine for feline hyperthyroidism—offer further opportunities for tailored treatment. Despite promising clinical outcomes in dogs and cats, broader application is limited by equipment costs, anesthesia requirements, and the need for specialized expertise. Future priorities include prospective clinical studies, refinement of species-specific dose constraints, and integration of RT with surgery, chemotherapy, and targeted therapies.

1. Introduction

Improvements in the husbandry and medical care of companion animals have led to longer lifespans for dogs and cats and, consequently, a greater clinical burden of neoplastic disease (Sarver et al., 2022). Cancer is now recognized as a leading cause of death in dogs and cats in the USA (Farrell et al., 2024), although robust epidemiological data from many regions—including Iran—remain limited (Abbaszadeh Hasiri et al., 2019).

Several tumor types in companion animals closely parallel those seen in humans, providing opportunities for translational insight (Surjan et al., 2011). In dogs, neoplasia ranges from benign to highly malignant lesions; skin tumors are among the most frequently diagnosed, followed by neoplasms of the gastrointestinal tract, mammary gland, urinary and reproductive systems, and the lymphoid and immune systems (Sarver et al., 2022).

The principal modalities for the management of

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solid tumors in veterinary practice are surgery, radiation therapy (RT), and chemotherapy. Surgery and RT primarily provide local–regional control, whereas chemotherapy—and, increasingly, targeted and immunotherapeutic approaches—address systemic disease. Despite cancer’s prominence in the public consciousness, RT and its applications in veterinary medicine remain comparatively specialized and unfamiliar to many general practitioners and owners (Gasyanova et al., 2017; Kent, 2017). Nevertheless, RT has become an integral component of multimodal cancer care for companion animals, with expanding indications for definitive, adjuvant, and palliative treatment of malignant tumors (Elliott, 2022).

Technical capabilities in veterinary radiation oncology have advanced substantially over the past two decades. Historical approaches that delivered dose with a single beam and relied on simple shielding to spare normal tissues have been superseded by conformal planning and delivery techniques. Contemporary practice increasingly employs dedicated small-animal radiotherapy platforms and medical linear accelerators with computed tomography–based planning, three-dimensional conformal radiotherapy, intensity-modulated and volumetric-modulated arc therapy, image-guided radiotherapy, and, where appropriate, stereotactic protocols that permit hypofractionation with steep dose gradients (Dunfield et al., 2018; Gasyanova et al., 2017; Vanhaezebrouck & Scarpelli, 2023). These innovations can improve tumor coverage and normal-tissue sparing, broadening the therapeutic window. Importantly, however, earlier technologies still retain advantages for selected indications and settings, and the guiding principle remains tailoring the technique to the clinical objective, patient factors, and resource context.

Clinical knowledge regarding outcomes and toxicity profiles of RT in dogs and cats has grown markedly, enabling more precise counseling about expected tumor control, acute effects, and late sequelae under both definitive and palliative fractionation schemes (Militi et al., 2024; Yoshikawa et al., 2023). By contrast, evidence for exotic and non-traditional species is sparse; in many such patients, current uncertainties still outweigh established knowledge, underscoring the need for prospective data collection and species-specific dose–response characterization. Practical considerations—analgesia requirements for immobilization, quality-assurance infrastructure, cost, and access to trained personnel—also shape real-world utilization.

This article provides an up-to-date overview of the applications and recent technological advances of RT in the treatment of cancer in companion animals. It highlights the expanding role of radiation within multimodal veterinary oncology and identifies gaps that warrant coordinated research efforts. Given ongoing investment in veterinary RT infrastructure, these de-

velopments are poised to further integrate radiation therapy into everyday clinical practice and improve outcomes and quality of life for animal patients.

2. Radiation Therapy in Companion Animals

Radiation therapy is primarily employed as a localized treatment modality in veterinary oncology. Its effectiveness in controlling tumor growth can vary substantially depending on the histological type of cancer, the tumor’s biological behavior, and the treatment protocol administered. In many cases, RT is capable of achieving durable local control and prolonging survival, particularly when applied under optimal clinical circumstances (Kent, 2017; Moore, 2002).

One of the most effective applications of radiation therapy is in the management of microscopic residual disease. Following surgical excision, if histopathology reveals incomplete margins or if the tumor is located in an area where complete resection is anatomically challenging, adjuvant radiation therapy is commonly administered to eradicate remaining malignant cells and reduce the likelihood of local recurrence (Grimes et al., 2023; Hildebrandt et al., 2025).

Radiation therapy may also serve as a definitive treatment when tumors are highly radiosensitive or when they are small and localized but unresectable due to anatomical constraints. In such cases, RT can be employed as a sole therapeutic modality, providing long-term local control (Hunley et al., 2010; Malfassi et al., 2022; Yoshikawa et al., 2023). Conversely, when tumors are extensive, unresectable, metastatic, or when surgery is declined or not feasible, RT is frequently administered with palliative intent. Palliative protocols are designed to alleviate clinical signs, such as pain, bleeding, or obstruction, thereby improving the animal’s quality of life even when cure is not possible (Fu et al., 2023; Militi et al., 2024; Tollett et al., 2016).

In addition, radiation therapy is often integrated into multimodal treatment strategies alongside chemotherapy. Chemotherapy may be administered systemically or by intralesional injection, depending on the tumor type and clinical context. The rationale for combining chemotherapy with radiation includes enhancing local tumor control through radiosensitization, addressing micrometastatic or disseminated disease, and improving overall treatment efficacy (Baja et al., 2022; Tellado et al., 2022; Woodruff et al., 2019). Such combined protocols are particularly valuable in aggressive or advanced-stage malignancies.

Overall, radiation therapy occupies a versatile role in veterinary oncology, functioning as an adjuvant, definitive, or palliative treatment, either alone or in combination with systemic therapies. Its application continues to expand as protocols are refined and evi-

dence on treatment outcomes accumulates, reinforcing its importance as a cornerstone of cancer management in companion animals.

3. Goals and Rationale for Radiation Therapy

The primary objective of radiation therapy (RT) in veterinary oncology is to deliver a cytotoxic dose of ionizing radiation to malignant cells while minimizing collateral injury to the surrounding normal tissues. Unlike conventional medical terminology that frequently emphasizes “cure,” oncologists typically frame therapeutic goals in terms of definitive intent or palliative intent, reflecting the clinical reality that complete eradication of cancer with no risk of recurrence or metastasis is uncommon (Kent, 2017; Vail et al., 2019).

3.1. Definitive Intent

Definitive radiation therapy is administered with the aim of achieving long-term local tumor control and prolonged survival. The treatment plan is based on the histopathological diagnosis, biological behavior of the tumor, and overall clinical evaluation of the patient. In some cases, RT may be prescribed preoperatively to reduce tumor size, thereby facilitating surgical excision, improving the feasibility of wound closure, and decreasing the risk of viable tumor cells being disseminated during surgery (Magalhaes et al., 2021; Yoshikawa et al., 2023). More commonly, RT is used postoperatively when incomplete surgical margins are suspected or confirmed, in order to eradicate microscopic residual disease and minimize the risk of local recurrence (Forrest et al., 2000).

Although surgical resection is often the preferred first-line option when feasible, there are many clinical contexts where radiation therapy offers significant advantages or may even represent the treatment of choice. These include large tumors that are not amenable to resection, neoplasms in surgically inaccessible or high-risk anatomical locations such as the brain, nasal cavity, head and neck region, vertebral column, or pelvic canal, as well as cases where surgical morbidity would be severe or disfiguring. Even when gross removal of a tumor is possible, microscopic extension of neoplastic cells beyond surgical margins is common, particularly in infiltrative tumor types, making adjuvant radiation a critical component of multimodal therapy (Mason et al., 2021; Zabielska-Koczywąg et al., 2017).

3.2. Palliative Intent

In contrast, palliative radiation therapy is directed toward alleviating clinical signs, reducing pain, and improving the quality of life in patients for whom long-term survival or cure is not a realistic expectation

(Fan et al., 2018). Palliative protocols typically employ lower total doses of radiation delivered in fewer fractions, with each fraction being larger in size compared to definitive schedules (Militi et al., 2024). This approach aims to provide meaningful symptom relief while minimizing the risk of acute or late radiation-associated toxicities (Rossi et al., 2019).

Palliative RT does not imply a lesser standard of care or therapeutic futility. Rather, it is a powerful intervention for managing pain, discomfort, or functional impairment caused by tumors that are unresectable, metastatic, or advanced at the time of diagnosis. By slowing tumor progression and reducing tumor-associated morbidity, palliative therapy can extend the time animals are able to live comfortably, thereby offering owners more quality time with their pets (Baja et al., 2022; Fu et al., 2023). In many cases, cancer can be managed akin to a chronic disease, where the therapeutic focus shifts from achieving cure to maintaining patient well-being and quality of life (Militi et al., 2024).

3.3. Combined Modalities

Radiation therapy may also be integrated with systemic therapies such as chemotherapy or targeted agents to optimize both local and systemic tumor control. The combination can enhance radiosensitivity of tumor cells, address micrometastatic disease, and improve overall therapeutic efficacy (Wustefeld-Janssens et al., 2021).

4. Types of Radiation Therapy in Veterinary Medicine

Several types of radiation therapy are currently available in veterinary oncology, including teletherapy, brachytherapy, plesiotherapy, and systemic radiotherapy using radionuclides. Among these, teletherapy and plesiotherapy are the most frequently employed in routine clinical practice for the treatment of malignant tumors in companion animals (Kent, 2017). These modalities differ primarily in the way radiation is delivered to the tumor.

4.1. External Beam Radiotherapy (Teletherapy)

External beam radiotherapy, or teletherapy, represents the most common approach to radiotherapy in veterinary medicine. It involves the use of a large external radiation source—most often a linear accelerator—to deliver high-energy radiation selectively to tumors that have been accurately localized with imaging, typically computed tomography (CT). In earlier years, orthovoltage and cobalt-60 machines were used; however,

modern practice now relies almost exclusively on linear accelerators (Moore, 2002; Tanabe et al., 2023).

Technological advances such as cone-beam CT (CBCT) have enabled image-guided radiotherapy (IGRT), which permits precise targeting of tumors located anywhere in the body while minimizing damage to adjacent normal structures (Søvik et al., 2010; Yuan et al., 2023). Furthermore, computerized treatment-planning systems are used to model dose deposition and optimize beam distribution, thereby improving tumor control rates and reducing radiation-induced complications. For central nervous system (CNS) tumors, magnetic resonance imaging (MRI) is frequently combined with CT to better delineate tumor boundaries, although MRI images alone cannot typically be used for dose calculation (Chiu et al., 2018).

4.2. Brachytherapy

Brachytherapy involves placing small radioactive sources directly within or adjacent to the tumor, either permanently (e.g., radioactive seeds) or temporarily (via catheters and afterloading devices). This technique provides a very high localized dose to the tumor while sparing surrounding normal tissue (Bloch et al., 2020). Although widely practiced in human oncology, brachytherapy is less common in veterinary medicine due to challenges in maintaining source stability, ensuring radiation safety, and the need to confine patients until the isotope decays or is removed. Nevertheless, when feasible, brachytherapy can achieve excellent local control with reduced collateral tissue damage compared with external beam radiotherapy (Vanhaezebrouck et al., 2025).

4.3. Plesiotherapy

Plesiotherapy is a related technique in which a radioactive source is applied directly onto the tumor surface rather than implanted within it. This approach is particularly useful for treating superficial lesions, such as certain skin tumors, where the tumor lies close to the surface and direct source placement ensures effective dose delivery with minimal exposure to underlying tissues (Goodfellow et al., 2006; Hammond et al., 2007).

4.4. Systemic Radiotherapy (Biologically Targeted Radiotherapy / Nuclear Oncology)

Systemic radiotherapy involves the administration of a radioactive therapeutic agent that localizes to tumor tissue through specific physiological or molecular processes. This approach, often referred to as biologically targeted radiotherapy or nuclear oncology, offers the potential to treat not only primary tumors but also distant metastases—an advantage over conventional radiotherapy, which is typically limited to local

or regional treatment (College of Veterinary Medicine, 2025; Peterson & Rishniw, 2021).

A well-established example in veterinary medicine is the use of radioiodine (iodine-131), which is the treatment of choice for feline hyperthyroidism caused by thyroid adenomas and is occasionally used for thyroid adenocarcinomas in dogs. Radioiodine therapy is highly effective because it delivers a relatively low dose with only short-term retention of radioactivity in the patient, making it both safe and practical. Higher doses, however, may be required when treating malignant thyroid carcinomas (McGovern et al., 2024; Morsink et al., 2022; Vanhaezebrouck et al., 2025).

Systemic radionuclides also hold promise for managing bone cancers. Bone-seeking radioisotopes, initially developed for humans with metastatic osseous neoplasia, have demonstrated utility in the treatment of both primary and metastatic bone tumors in dogs and cats. In addition, targeted agents such as monoclonal antibodies and small-molecule isotope carriers—already in clinical use for neuroendocrine and mammary carcinomas in human oncology—are emerging as potential tools in veterinary practice (Barca et al., 2021; Barnard et al., 2007; Selting et al., 2023).

As the range of biologically targeted radiopharmaceuticals expands, systemic radiotherapy is expected to play an increasingly important role in veterinary oncology, potentially broadening the spectrum of tumors amenable to treatment and improving long-term outcomes for companion animals.

5. Emerging Techniques and Future Directions

5.1. Electron Radiotherapy

Linear accelerators are capable of generating both high-energy X-rays and electron beams, typically within the energy range of 4–20 MeV. Whereas X-rays are primarily employed for the treatment of deep-seated tumors, electron beams are more suitable for neoplasms located within the skin and subcutaneous tissues. Modern linear accelerators are designed to deliver both photons and electrons by either scattering accelerated electrons or directing them onto a target within the machine (Arunkumar et al., 2010; Strydom et al., 2006).

One of the major advantages of electron therapy lies in its controllable depth of penetration, which reduces unnecessary irradiation of normal tissues beyond the target volume. For instance, a 6-MeV electron beam can effectively deliver radiation up to a depth of 2 cm, with minimal dose beyond 3 cm. This makes electron beams particularly valuable for the treatment of superficial or thoracic wall tumors. The ability to tailor beam energy provides veterinary oncologists with flexibility in matching dose distribution to tumor

depth, thereby improving local control while sparing surrounding structures (Chao et al., 2009; Gerbi et al., 2009; Strydom et al., 2006).

5.2. Intensity-Modulated Radiotherapy (IMRT)

Intensity-modulated radiotherapy (IMRT) represents one of the most significant technological advancements in veterinary oncology. By modulating beam fluence during delivery, IMRT allows for highly conformal dose distributions that closely match the three-dimensional geometry of the tumor. This results in steep dose gradients at field margins, effectively reducing exposure to adjacent healthy tissues (Meier et al., 2021; Van Asselt et al., 2020).

IMRT requires advanced treatment planning software, multileaf collimators, and on-board imaging to achieve submillimeter precision. While this technique is resource-intensive and demands strict patient positioning, its clinical benefits include reduced toxicity, increased sparing of critical organs, and the possibility of dose escalation to resistant tumors. As availability of IMRT expands in veterinary oncology centers, it is expected to become a cornerstone of precision cancer therapy for companion animals (Dolera et al., 2018; Meier et al., 2021; Van Asselt et al., 2020).

5.3. Strontium-90 Plesiotherapy

Strontium-90 (^{90}Sr) is a long-lived beta emitter with a half-life of approximately 29 years and an emission energy of 2.27 MeV. Its limited penetration makes it particularly effective for treating superficial neoplasms. In veterinary medicine, ^{90}Sr has been successfully applied to oral and nasal planum squamous cell carcinoma and cutaneous mast cell tumors in cats, conjunctival squamous cell carcinoma in dogs and horses, and select cases such as lingual plasmacytoma, limbal melanoma, and hemangiosarcoma in dogs (Goodfellow et al., 2006).

The therapeutic advantage of ^{90}Sr lies in its ability to deliver high doses directly to the tumor surface with minimal exposure to deeper normal tissues. While its clinical use is somewhat limited compared to external beam approaches, it represents a valuable option for small, localized, and superficial lesions where conventional radiotherapy would be less effective or overly invasive (Goodfellow et al., 2006; Radioisotopes & No, 2015; Turrel et al., 2006).

5.4. Stereotactic Radiosurgery (SRS)

Stereotactic radiosurgery (SRS) is a specialized form of high-precision radiotherapy in which very large doses are delivered in a single session using a linear accelerator equipped with image-guided systems. This approach employs very narrow margins around the tumor

and aims to achieve complete ablation of the lesion (Dunfield et al., 2018).

Although widely applied in human neuro-oncology, SRS remains relatively new in veterinary medicine, with only a limited number of published clinical reports. Its potential advantages include curative treatment of small, well-defined tumors in critical locations, particularly in the brain and spine, where surgery carries significant risks (Rohrer Bley et al., 2024).

5.5. Stereotactic Radiotherapy (SRT)

Stereotactic radiotherapy (SRT), sometimes referred to as stereotactic body radiotherapy (SBRT), extends the principle of stereotactic targeting to multiple treatment sessions—typically three to five fractions. Large, conformal doses are sculpted to the tumor volume with submillimeter accuracy, minimizing toxicity to adjacent structures (Rohrer Bley et al., 2024).

Treatment planning requires advanced imaging, usually CT in combination with MRI for central nervous system tumors, and strict immobilization to ensure reproducibility. The capacity to deliver high biologically effective doses in a limited number of sessions makes SRT an attractive option for tumors in anatomically challenging locations, offering both local control and preservation of quality of life (Dunfield et al., 2018).

5.6. Radioactive Implants (Brachytherapy)

The implantation of radioactive isotopes such as cobalt-60, cesium-137, radium, and iridium-192 in the form of needles or seeds represents another therapeutic modality, although its use in veterinary oncology has historically been limited. These sources can be permanently or temporarily implanted within the tumor, delivering high radiation doses locally with relative sparing of surrounding tissues (Northrup et al., 2004; Wilkie & Burt, 1990).

Brachytherapy has shown clinical promise in treating accessible malignancies, such as malignant perianal tumors in dogs and squamous cell carcinomas of the eyelids in horses and cattle (Bloch et al., 2020). Despite logistical challenges, including radiation safety and technical expertise, advances in afterloading devices and isotope encapsulation may facilitate broader clinical application in veterinary patients in the future.

6. Discussion and Conclusion

Radiation therapy has established itself as an essential modality in the management of neoplasia in companion animals, particularly dogs and cats, and continues to expand in scope as new technologies and clinical evidence emerge. Numerous studies and clinical experiences have demonstrated its ability to provide

durable tumor control, improve quality of life, and, in some cases, extend survival. Beyond traditional small animal patients, there is growing recognition that radiation may also offer therapeutic benefits in exotic and non-traditional species; however, systematic data on these groups remain scarce. To fully realize its potential, clinicians must continue to define optimal treatment protocols, dose constraints, and scheduling strategies tailored to the unique physiology of each species.

Veterinary radiation oncology has advanced markedly over the past two decades, paralleling developments in human oncology. The introduction of conformal treatment planning, image guidance, intensity-modulated radiotherapy (IMRT), stereotactic techniques, and the integration of advanced imaging modalities has revolutionized therapeutic precision. These innovations have enabled the safe delivery of higher tumoricidal doses while minimizing exposure to adjacent normal tissues, thereby reducing acute toxicities and long-term complications. In particular, IMRT has demonstrated the capacity to significantly lower radiation-associated side effects, while stereotactic radiotherapy (SRT) and stereotactic radiosurgery (SRS) offer the possibility of hypofractionated treatment courses with minimal toxicity, making therapy more convenient for both patients and owners. Image-guided radiation therapy (IGRT) further extends treatment capabilities to anatomical sites that were previously not considered feasible for radiotherapy.

Nevertheless, important challenges remain. Traditional fractionated radiotherapy continues to serve as the standard of care for many tumor types, and for many practices remains the most accessible form of treatment due to its established efficacy and wider availability. However, access to advanced modalities is often constrained by infrastructure requirements, costs, and the need for highly trained personnel. Furthermore, despite the growing body of literature, questions persist regarding the most appropriate clinical indications, long-term outcomes, and quality-of-life considerations following treatment. As more patients experience extended survival due to improved therapeutic efficacy, managing late effects of radiation and optimizing survivorship care will require increasing attention.

Future research should prioritize multi-institutional collaboration to strengthen the evidence base through prospective studies, long-term follow-up, and species-specific dose–response data, particularly in exotic animals where knowledge gaps remain substantial. Equally important is the continued integration of radiation therapy into multimodal treatment regimens that include surgery, chemotherapy, and emerging systemic and targeted approaches. Such strategies promise not only improved local tumor control but also better systemic management of cancer.

In conclusion, radiation therapy has matured from

a specialized and relatively underutilized tool into a cornerstone of veterinary oncology. With the continued adoption of advanced technologies, refinement of treatment protocols, and expansion of clinical research, radiotherapy is poised to further enhance outcomes, extend survival, and, most importantly, improve the quality of life of companion animals affected by cancer.

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ORIGINAL RESEARCH PAPER

A Comprehensive Review of Microneedling and Facial Skincare Strategies in Anti-Aging Dermatology

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Abstract

These days, there are different techniques for skin rejuvenation. One of them is microneedling, which can improve skin health with minimal side effects and good results. Microneedling is an effective skincare method for facial rejuvenation. It is a good source of growth factors, cytokines, and other biologically active substances that can enhance rejuvenation and wound healing processes. During the microneedling method, all these substances are obtained from the patient's blood without any pain and have the advantage of reducing the immune response, making the procedure safer and better tolerated, with minimal side effects and lower cost. Therefore, it is a useful approach for skin anti-aging. It is currently being used extensively for facial rejuvenation. In this process, using a combination of microneedling, mesotherapy techniques, and facial skincare for treating acne scars, melasma, and facial scars is more powerful. So, in this article, the microneedling method and its remarkable effects on skin anti-aging and reducing skin problems will be studied.

1. Introduction

Skin aging includes some skin problems like pigment changes, wrinkles, thinning, and loss of elasticity due to both genetic and environmental factors. A variety of medical procedures and topical cosmetics are used to treat different signs of aging. Microneedling is a relatively new, minimally invasive procedure that can create tiny holes in the surface of the skin in a controlled manner and depth, by rolling and penetrating different layers of skin with its tiny needles. It quickly became widely popular and accepted because it requires minimal training and is a simple, inexpensive, safe, and effective technique for all types of skin and most skin issues. These days, microneedling is being used as a

collagen induction therapy for facial scars and skin rejuvenation more than before. It is now also commonly used as a transdermal delivery system for therapeutics and vaccines instead of some injections that have a lot of side effects for the skin in the near future. The emergence of the concept of microneedling dates back to 1995, when Orentreich described dermal needling in a subcision format for scar treatment, and then, when a plastic surgeon used a tattoo gun in 1997 and independently described it by Kamiland (Buzalaf and Levy, 2022; Orentreich and Orentreich, 1995; Camirand and Doucet, 1997). In 2006, Fernandez, as a surgeon, designed his drum-shaped device with several thin protruding needles and used it for transcutaneous collagen induction for skin (Amit, 2013; Fernandes, 2005).

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2. Skin Aging

Skin aging is a visible sign of physical aging (Yaar *et al.*, 2002). As collagen and elastic fibers decrease in the skin, wrinkles appear gradually over time (Gilchrest, 1989), and with the loss of fat and skin moisture, the process of skin aging accelerates (Yaar and Gilchrest, 2001). Therefore, there is a growing demand for surgical treatments and skin rejuvenation. So, it is better to have some useful non-surgical treatments to reduce the percentage of unnecessary surgeries and their side effects. Its usefulness is determined by minimal invasiveness and downtime. Currently, different types of non-invasive cosmetics are available on the market. However, limited efficacy is often due to low skin penetration of active compounds, as the stratum corneum acts as an external barrier. This protocol is within the principles of good clinical practice and applicable regulatory requirements. The Institutional Review Board of the Human Research Ethics Committee of Thammasat University (Medicine) approved all study protocols (number: MTU-EC-OO-0-078/64, November).

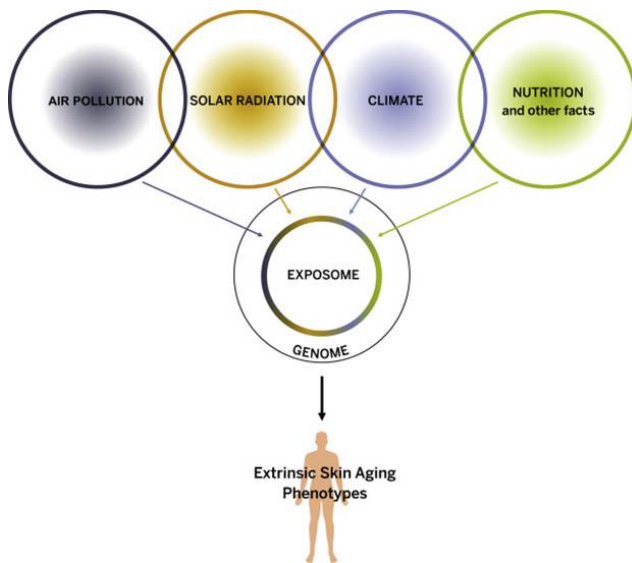


Fig. 1. Different external factors can affect skin cells and cause skin aging, and in some conditions, can increase the speed of skin aging (Krutmann *et al.*, 2021).

According to one of the references that showed good results, the microneedle patch and HA were applied to the right side of the patients, and the microneedle patch only was applied to the left side. Each treatment was applied to the nasolabial folds for 8 weeks. The density of the microneedle array in this study was 265 needles/cm, and the shape of the microneedle patch was designed to cover the nasolabial fold area. Based on these specifications, the total area of the microneedle array within the patch was 7.5 cm. The fins of each four-pronged star-shaped microneedle formed a gap or channel between the groove of the microneedle and the tissue when penetrated into the skin. The

reservoir was applied only to the contralateral side every 2 weeks for a total of 8 weeks. All applications were performed after washing the face, and no local anesthetic was administered during the clinical trial. Participants were instructed to massage both sides of the patch for 5 minutes before removing it. Moisturizer was provided to each participant. The drug or solution delivery mechanism of the four-pronged star microneedle array was fabricated on a tissue substrate. In this method, the image shows hyaluronic acid solution being forced through a syringe connected to a microneedle patch. According to the patient characteristics, the participants in this study had a mean age of 50.78 ± 6.41 years. Of the total respondents, 9 were 40 years old and below, constituting 39.13%; 12 were 50 years old and below, constituting 52.17%; and 2 were 60 years old and below, constituting 8.69%. All participants were female.

3. Intrinsic and Extrinsic Skin Aging Processes

The aging of the skin involves gradual deterioration. This is because of both intrinsic and extrinsic skin aging processes. Intrinsic aging is a natural function, a progressive physiological process, and can cause the formation of fine lines, thinning, and dryness in the skin structure. In addition to all these, gradual skin atrophy can be seen. Extrinsic aging is caused by the influence of external environmental factors such as ultraviolet (UV) light, infrared radiation, environmental pollution, smoking, malnutrition, and psychological stress. These items can lead the skin to a loss of skin elasticity, which increases sagging and the formation of coarse wrinkles (Wadstein *et al.*, 2022; Zhang and Duan, 2018). External factors also appear to reduce the levels of antioxidants in the skin texture and can increase the levels of reactive oxygen species (ROS) in the skin. A middle-aged woman whose skin shows signs of aging most of the time may present with hyperpigmentation, erythema, telangiectasia, wrinkles, and localized dryness (Kammeyer and Luiten, 2015).

4. Skin Microenvironment

Currently, the evidence suggests that changes in the skin microenvironment can significantly improve wrinkles, pores, and elasticity in the combination group compared to MN alone. A recent study concluded that skin structural remodeling in skin biopsy pathology can be observed primarily at the junctional side (El-Domyati *et al.*, 2020). Additionally, epidermal histology shows a significant increase in epidermal thickness on both the skin needling side and the combined side (i.e., skin needling and mesenchymal stem cell-derived amniotic fluid). It became clear that this is consis-

tent with current results, showing that improvement in skin rejuvenation was greater on the combined side than on the MN-alone side. This difference may be due to differences in microneedle length (0.25 mm in the Korean study and 0.5 mm in another target study) (Cassiano *et al.*, 2019). According to references and previous studies, MN single treatment of melasma with a needle length of 0.5 mm significantly reduced melanin density, pendulous melanocytes, and pathological basement membrane (Cassiano *et al.*, 2019). These studies provide strong evidence for effective treatments. Therefore, this method will be used successfully for facial anti-aging. Data from papers show that combination therapy with MN and MSC derivatives can have significant improvement over MN alone. In summary, the combination of MN and hUC-MSCs-CM is a safe and effective treatment for facial rejuvenation and may be used as a new method for anti-aging therapy in recent years (Cassiano *et al.*, 2019).

5. Physiological Parameters of Skin Water Content and TEWL

Maybe it raises a question: What are the water content and TEWL in the skin? They are indicators of skin barrier function. After treatment, there were no significant changes in hydration content between the

MN with hUC-MSCs-CM side ($P=0.06$) and the MN-alone side ($P = 0.12$). There was no comparison with the baseline, and skin elasticity was significantly improved in both groups ($P=0.00$). There were no side effects during the study period. Cell therapy is a kind of skin rejuvenation method using extracellular vesicles and stem cells such as MSCs and BMSCs due to their ability to repair and regenerate tissues and organs in cosmetic and reconstructive surgery (Zarei and Abbaszadeh, 2019; Suh *et al.*, 2019). Effects of conditioned media derived from BMSCs or adipose tissue and the impacts of derived stem cells on skin rejuvenation have been demonstrated (Kim *et al.*, 2021; Li *et al.*, 2019). Skin brightness and texture are more closely related to skin rejuvenation (the less pigment in the skin and the lighter the color). Therefore, vascular permeability is needed to improve tissue nutrition.

6. Skin Moisture and Elasticity

Skin moisture and elasticity were assessed using a Cutometer (Ahn *et al.*, 2007), which represents the overall elasticity of the skin, including viscous deformation. It showed no statistically significant improvement ($p=0.098$). Furthermore, the water content of the skin, called skin viscoelasticity, did not show statistically significant improvement throughout the main study ($p=0.205$).

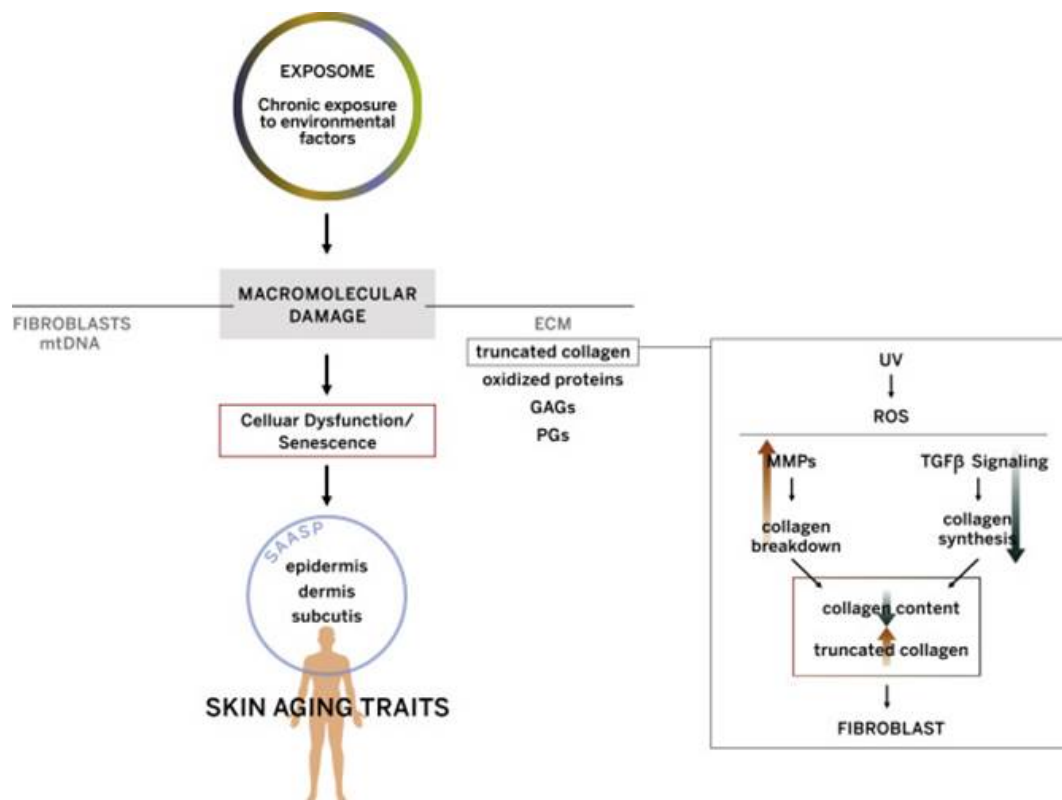


Fig. 2. According to all skin aging conditions, external factors can penetrate into the skin layers and cells (like UV) and affect the core and its DNA. These factors can cause changes in cells like fibroblasts, and subsequently, problems can be seen in the levels of ROS, MMPs, and TGFβ (Krutmann *et al.*, 2021).

According to references, some skin features like wrinkles, roughness, and pore volume improved after the first treatment session. Wrinkles improved by 9.12% positive changes, according to data measured with the Antera 3D Image Capture System. The degree of improvement in wrinkles was clearly significant after the first and second treatments and continued with positive changes without additional treatment sessions. Overall, wrinkles improved by 11.72% positive changes. Wrinkle improvement was statistically significant ($p < 0.001$) throughout the study (Akhtar *et al.*, 2011).

7. Factors for Improving Skin Rejuvenation

7.1. Endothelial Progenitor Cells and Cytokines

Endothelial progenitor cells (EPCs) differentiated from human embryonic stem cells (hESCs) showed improved blood perfusion of injured tissues and secreted high levels of growth factors and cytokines to the main sections (Cho *et al.*, 2007; Lee *et al.*, 2011b). Conditioned medium (CM) of hESC-derived EPCs (hESC-EPCs), containing multiple growth factors and cytokines, significantly enhanced the proliferation and migration of Mal-fibroblasts and epidermal keratinocytes, as well as increased collagen synthesis in fibroblasts (Lee *et al.*, 2011b). In this regard, growth factors help reduce the signs of aging (Fitzpatrick and Rostan, 2003). Some growth factors exhibit skin whitening effects by inhibiting melanin production (Kim *et al.*, 2008). However, recently, the beneficial roles of growth factors in skin rejuvenation have only been investigated (Velugotla *et al.*, 2017; Kim *et al.*, 2009a; Park *et al.*, 2008), and no controlled clinical trials have been conducted. Hydrophilic molecules larger than 500 Da have poor penetration into the stratum corneum (Bos and Meinardi, 2000; Jakasa *et al.*, 2007). Most growth factors are large hydrophilic molecules larger than 20 kDa. Therefore, they are unlikely to penetrate the epidermis in measurable amounts and cause pharmacological effects. Microneedling was used to improve skin penetration of hESC-EPC CM, and it was successful. Triplex cytokine analysis is a useful method for analyzing CM, and it showed that hESC-EPCs highly expressed several growth factors including fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), fractalkine, and granulocyte-macrophage colony-stimulating factor. It has become clear that this is truly occurring. Of course, it stimulates factors like interleukin-6 (IL-6), GM-CSF, vascular endothelial growth factor (VEGF), platelet-derived growth factor AA (PDGF-AA), and PDGF-BB, which is involved in fibroblast proliferation and regulates cell growth. VEGF promotes skin angiogenesis and can increase vascular permeability and

improve tissue nutrition (Fabi and Sundaram, 2014).

8. Recent Treatments for Rejuvenation

Combating signs of aging such as wrinkles, enlarged pores, decreased resistance, and irregular pigmentation, and rejuvenating the skin is one of the biggest challenges of the 21st century (Boismal *et al.*, 2020). Recently, mesenchymal stem cells (MSCs) and microneedling (MNs) have been widely used for anti-aging treatments. The purpose of this study is to evaluate the efficacy of the combination of MN and conditioned medium (hUC-MSCs-CM) and human umbilical cord-derived mesenchymal stem cells in skin brightening and rejuvenation, which are the most important factors in the anti-aging process. Skin aging can be influenced by both intrinsic (chronological) and extrinsic (environmental) factors, leading to deterioration in appearance and loss of function (Choy and Prausnitz, 2011). Skin aging not only affects the physiological functions of the skin but also impacts people's psychology and social life. As a result, several non-surgical treatments have been developed in recent years to combat skin aging, including oral treatments, ointments, dermabrasion, chemical peels, and laser treatments. However, these may be associated with prolonged recovery, hyperpigmentation, and scarring. Microneedling (MN) is widely used. Microneedling (MN) is widely used as a treatment for skin diseases and skin rejuvenation because of its safety and effectiveness. There are two main reasons for its medicinal use: (a) MN can promote the skin's natural healing process more than alone, and it is exciting and hopeful. It can penetrate the epidermis and papillary dermis layer (Yang *et al.*, 2021) and form pores, so it can start the skin's repair mechanisms better than other processes (Soliman *et al.*, 2018). Also, this can lead to short-term aggregation of inflammatory cells, fibroblast proliferation, long-term remodeling, and collagen and elastin synthesis (Yang *et al.*, 2021). (b) MN can improve drug penetration; this is due to penetrating the stratum corneum barrier (Choy and Prausnitz, 2011). Therefore, it is difficult for drugs to be absorbed through the skin layers. MNs create small temporary holes that penetrate the stratum corneum barrier over a short period of time (Gupta *et al.*, 2011). According to the reference, before the treatment in the MN method, all participants' faces had to be cleaned with facial cleanser. MN treatment was performed in eight rows using a total of 192 needles with a length of 0.5 mm (Gupta *et al.*, 2011).

9. Microneedling Devices

- Dermaroller
- Dermapen

9.1. Dermaroller

A standard medical dermaroller has a 12 cm long handle with a 2×2 cm wide drum-shaped cylinder at one end, which consists of 192 thin microneedles (usually 0.5 to 3 mm) arranged in 8 rows and 24 circular arrays. The length and diameter are 0.1 to 0.25 mm, and it is not an automatic device (Amit, 2013).

9.2. Dermapen

Dermapen is an automatic microneedling device that looks like a pen. This ergonomic device uses disposable and sterile needles and guides to adjust the length of partial mechanical resurfacing needles. Nine to twelve needles are arranged in a row at the tip. It uses a rechargeable battery and operates in two modes: fast mode (700 cycles/minute) and slow mode (412 cycles/minute), which vibrates like a stamp on the skin in each mode (Arora and Gupta, 2012). The needle tip is hidden in a guide, making this process safe and comfortable in tight areas such as the nose. In this method, eyes and lips can be treated without damaging adjacent skin, and this is a really positive point for this method. This makes the procedure less painful and more economical, as there is no need to purchase new instruments each time. This technique was developed to overcome the problems of pressure application and resulting penetration depth. So, all the pressure and depth depend on the part of the skin that is selected for treatment and repair.

10. Microneedling

Microneedling, also known as percutaneous collagen induction (PCI), is a minimally invasive technique that was first described in principle by Orentreich (Orentreich and Orentreich, 1995). It is a subcutaneous excisional procedure that removes the skin beneath recessed scars and wrinkles and stimulates collagen in the body. Fernandes used a similar technique of inserting a 15-gauge needle into the skin beneath the wrinkle (Fernandes and Signorini, 2008; Fernandes, 2005). Fernandez's technique was developed at that time and then followed by the dermal roller (Fernandes and Signorini, 2008). The dermal roller is a sterile plastic cylinder with stainless steel needles protruding 1 to 3 mm from the surface of the cylinder. The skin roller is forcefully rolled over the skin, creating numerous needle pricks and resulting in the formation of thousands of microscopic wounds in the dermis, which triggers the natural inflammatory response after trauma (i.e., growth factor release). As a result, formation of collagen and elastin occurs (Fernandes and Signorini, 2008; Fernandes, 2005). The latest automated microneedling devices are replacing dermal rollers. The needle barrel is typically replaced with a

sterile disposable needle cartridge with a variety of different needle configurations for different parts of the skin with different conditions. The automated device allows the operator to define the puncture depth and frequency of needle punctures and control the treatment area and coverage, rather than relying on the operator physically rolling the device over the skin. Currently, needles of automated microneedling devices consist of several thin sterile needles, typically 0.5 to 1.5 mm in length. In addition to age-related skin conditions (wrinkles, laxity, etc.), there is a growing list of skin conditions that respond to PCI therapy. This list includes skin issues like acne scarring, hyperpigmentation, alopecia, hyperhidrosis, and PCI. It has also been reported that it is being used in transdermal drug delivery systems. However, most studies on microneedling are case series or small randomized controlled trials (Hou *et al.*, 2017). In this paper, the research objective was to assess the effectiveness and outcome of best practices such as microneedling for repairing or treating various skin afflictions and diseases and to establish safety precautions for facial skin renewal and rejuvenation with percutaneous collagen injection. Volunteers who were on anticoagulant therapy or taking aspirin or high-dose non-steroidal anti-inflammatory drugs (NSAIDs) in the previous 14 days were excluded. Further exclusion criteria were: hepatitis, active acne vulgaris on the face, inflammatory skin disease, uncontrolled diabetes, keloid scars, human papilloma virus (HPV), birthmarks, eczema, known malignancy, chemotherapy, radiation therapy or high-dose corticosteroid treatment, known allergy to local anesthetics, and past treatment with filler injections or neuromodulators within 3 months (Hou *et al.*, 2017).

11. Microneedling Delivery Systems

Microneedle delivery systems provide a minimally invasive and painless transdermal drug delivery method that is particularly useful for vaccines. An insulated needle is used to penetrate the skin and emit high-frequency current from the tip of the needle, without damaging the overlying epidermis (Cohen and Elbuluk, 2016). The needle depth can be adjusted from 0.5 mm to 3.5 mm, allowing careful targeting of different layers of the dermis (Chandrashekar *et al.*, 2014). Microneedling Radio Frequency Technology (MNRF) is safe for all skin types because it does not damage the epidermis. So, it is a good method for treating scars, hyperhidrosis, skin tightening, and rejuvenation.

12. Effects of Microneedling on Skin

When a dermaroller is applied to the skin with its 192 needles, 2 mm long and 0.07 mm in diameter, and applied 15 times to that section of the skin, various pro-

teins, potassium, and growth factors are released from the cells. As a result, fibroblasts migrate to the injury site and collagen is induced. Keratinocyte hyperproliferation is downregulated by microneedle treatment in acne patients to balance overall cellular activity (Liebl and Kloth, 2012). Microneedling improves the delivery of various drugs across the skin barrier by bypassing the stratum corneum and delivering drugs directly to the vascularized dermis.

It was also shown that the follicular infundibulum was significantly dilated by 47%, which may partially explain the increased drug penetration. Additionally, it removes scale and sebum residue around the infundibulum area (Serrano *et al.*, 2015). Micropuncture is performed using microneedling, and depending on the applied pressure, holes per square centimeter reach the papillary dermis layer (Serrano *et al.*, 2015). Each pass creates 16 micropunctures per square centimeter in the stratum corneum without major damage to the epidermis (Nair and Arora, 2014). These micro-injuries result in minimal superficial bleeding and trigger a wound healing cascade, releasing various growth factors including connective tissue activation protein, connective tissue growth factor, and fibroblast growth factor (FGF) (Freinkel and Woodley, 2001). Additionally, needles dissolve old and hardened scar strands and allow the texture to undergo revascularization, angiogenesis, and collagenogenesis. These are initiated by fibroblast migration, proliferation, and intercellular matrix deposition (Fabbrocini *et al.*, 2009). A fibronectin matrix is formed after 5 days of injury, which determines collagen deposition and results in skin formation. The tightening effect lasts for 5 days, and collagen III forms for 7 years. The depth of neocollagen with a 1.5 mm needle was found to be 5–600 μm . Histological examination of skin treated with 4 microneedling sessions, spaced 1 month apart, showed up to a 400% increase in collagen and elastin deposi-

tion at 6 months postoperatively and an increase in the stratum spinosum at 1 year postoperatively. A thick, normal rete-canthus was observed (Aust *et al.*, 2008a). The collagen fiber bundles appear to have a regular lattice pattern rather than parallel bundles like in scar tissue (Nair and Arora, 2014). Libre *et al.* proposed an alternative hypothesis to explain how microneedling works (Liebl and Kloth, 2012). The resting potential of the cell is approximately -70 mV, but as the needle approaches the membrane, the internal potential increases rapidly to -100 mV. This function increases cellular activity and releases various proteins, growth factors, and potassium from the cells, leading to fibroblast migration and collagen induction (Majid *et al.*, 2014; Liebl and Kloth, 2012; Kloth, 2005). Keratinocyte hyperproliferation is downregulated by microneedle treatment in acne patients to balance the overall cellular activity (Liebl and Kloth, 2012). As a result of using microneedling, it improves the delivery of various drugs across the skin layers by bypassing the stratum corneum and delivering drugs directly to the vascularized dermis. Additionally, it also removes scale and sebum residue around the infundibulum (Serrano *et al.*, 2015). Microneedling is a simple in-office procedure that takes 10 to 20 minutes, and the process depends on the area being treated. Before the procedure, patients must receive counseling that explains the expected results, delayed response, and the need for multiple sessions for optimal outcomes. At this time, for maximizing collagen formation in the skin, it is advisable to prepare the skin preoperatively with vitamin A and vitamin C preparations twice a day for at least one month. According to references, it is important to know that vitamin A affects 400–1000 genes that control the proliferation and differentiation of all major cells in the epidermis and dermis, and vitamin C is essential for normal collagen production (Aust *et al.*, 2008a).

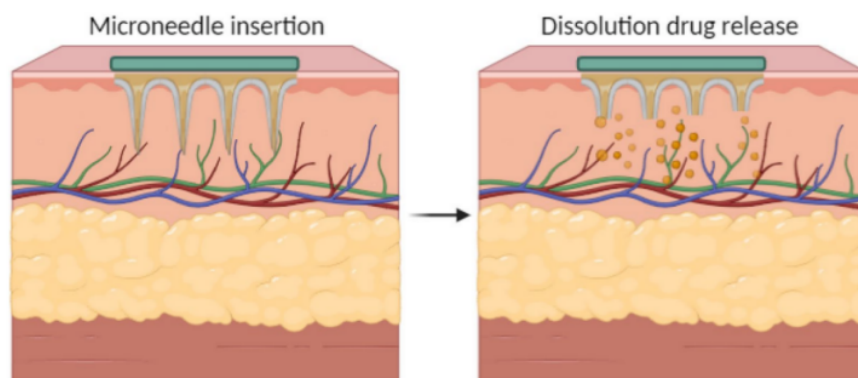


Fig. 3. After performing microneedling on skin tissue, the needles create pores in the skin, allowing drugs to be released more easily into deeper layers. Blood vessels can absorb the drug effectively. So, it is a good method for drug delivery systems (Dave *et al.*, 2024).

13. Microneedling Function

During the procedure, by driving microneedling into the skin, microscopic wounds are formed within the papillary dermis, causing the normal chemical cascade that follows trauma. This includes an inflammatory phase with the release of cell growth factors, followed by a proliferative phase, and finally a remodeling phase with stimulation of collagenogenesis and the formation of a stronger collagen matrix for repairing the skin texture. References showed improvement in facial wrinkles and skin texture at the end of its 90-day treatment with microneedling, but the largest (statistically significant) effect was observed on day 150. This improvement in facial wrinkles demonstrates the cumulative effects of transdermal collagen induction and ongoing dermal collagen remodeling (Fernandes and Signorini, 2008; Fernandes, 2005). Improved skin laxity is primarily a result of collagen III and collagen I quantity. One of the most important changes in this process is skin tightening. This process takes up to 12 months, so it is not surprising that statistically significant results were not obtained until day 150 of the study. Future studies would benefit from a preliminary follow-up of 120 days and an extended follow-up of 6 to 12 months to fully exploit the potential of this technology. On the other hand, statistically significant improvements in skin texture were observed at the end of treatment on day 90, due to the conversion of collagen III to collagen I and subsequent skin tightening. Additionally, epidermal remodeling is expected to be more measurable in the early stages of PCI treatment than in the later stages. Wrinkle evaluation results and skin texture improvement were confirmed by 3D shape measurement in the reference. As expected, the observed improvement in the common roughness index appeared to be greater than the observer rating measurements (Fernandes and Signorini, 2008; Fernandes, 2005). This is consistent with data previously reported by Friedman and Fujiwara. However, the results provide evidence of the feasibility and usefulness of 3D profilometry in obtaining a skin topography with higher resolution than clinical examination alone, supporting the use of 3D profilometry in clinical evaluation. It is argued that this measurement indicates adaptive improvements that may not be detected by expert evaluators. According to the reference, the epidermis was structurally relatively healthy and the basement membrane was not damaged during PCI treatment with the study device (Aust *et al.*, 2008b). Side effects associated with the use of this technique were well managed. As a result, the treatment effect was adequate and well tolerated by patients. No serious adverse events were reported. No abnormal skin observations were recorded by physicians after treatment or during the treatment session, and the subjects themselves did not report any such issues. Of these, no cases

of hyperpigmentation, transient or otherwise, were reported. That is, only in some references, the number of melanocytes remained constant in subjects who received microneedle treatment. Studies on skin condition after microneedling treatment were conducted by Aust (Aust *et al.*, 2008b) and Bonati (Bonati *et al.*, 2017).

14. Microneedling and Growth Factors

The use of growth factors in microneedling, according to skin texture functions, has shown certain results regarding collagen remodeling. In a case series, the use of topical growth factors on photodamaged skin was reported in 8 patients who received a total of 3 treatments with microneedling, spaced 10 days apart. The growth factor gel contained epidermal growth factor, fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor. An independent physician clinical evaluation using the Fitzpatrick Wrinkle Scale showed significant improvement in skin texture, fine lines, and wrinkles (Pamela, 2018). Another study examined 25 patients in a split-face randomized controlled trial and concluded that dermaroller microneedling was associated with growth factors (Lee *et al.*, 2014). In a split-face study involving 10 patients, El-Domyati *et al.* (El-Domyati *et al.*, 2020) investigated the use of their manual dermaroller using amniotic fluid-derived stem cells on acne scars. Although serum itself does not contain growth factors, stem cell therapy stimulates paracrine secretion of growth factors, extracellular matrix molecules, and interleukins. After performing five sessions spaced one month apart, the authors observed improvement in the histological structure of collagen and elastin, as well as statistically significant histological and clinical improvement. Another study using 12 human embryonic stem cells with flat microneedling (0.25 mm roller) showed improved pigmentation and wrinkles by physician and software analysis after five sessions at 2-week intervals. This study showed statistically significant improvement in 13 dermatological evaluation parameters, including uniformity of skin tone, melanin index, texture, and firmness. After that, clarity evaluation by subjects was observed in control subjects. Some studies have shown that microneedling and growth factors significantly improve skin texture, fine lines, wrinkles, pigmentation, and acne scarring, as well as histological changes in epidermal thickness and scar appearance. Clinical improvement has been shown in the results. Further studies are needed to characterize the effects of topical growth factors as adjuvants in dermatological procedures.

15. Microneedling and Skin Aging

Topical application of growth factors via microneedling has the potential to ameliorate signs of aging. Clinical symptoms of intrinsic and extrinsic skin aging include changes in pigment, thinning of the skin, formation of wrinkles and fine lines, loss of moisture and elasticity. Growth factors act as chemical messengers that mediate cell proliferation, repair processes, and ECM formation. Microneedling creates controlled micropunctures in the skin that induce collagen formation, angiogenesis, and wound healing. Thus, anti-skin aging functions of microneedling can help the skin texture increase its ability to remain healthy and youthful (Schaefer and Lademann, 2001).

16. Microneedling and Collagen

Microneedling, also known as collagen induction therapy, is a common skin therapy that uses a device to penetrate the dermis to an even depth and create controlled skin damage (Sun *et al.*, 2024). This injury induces rapid healing, which then stimulates the production of collagen and elastin fibers, resulting in skin remodeling and rejuvenation (Iriarte *et al.*, 2017; Wu *et al.*, 2020). However, in recent years, the microneedling device has been combined with the delivery of radio-frequency energy to heat the underlying skin layers, improving skin remodeling and clinical outcomes (Kim *et al.*, 2013). Microneedling is generally considered a safe and inexpensive alternative to other forms of skin rejuvenation. Local anesthetic is usually used before the procedure to minimize discomfort. In a recent review, the most common side effects associated with this treatment include temporary pain or discomfort, erythema, and edema (Juhasz and Cohen, 2020). These effects are mild and self-limiting but can cause concern about the patient's appearance. Therefore, this method can increase healthy collagen levels, and due to its minimal side effects, it is a highly effective way to help the skin maintain optimal collagen levels and remain in a healthy state.

17. Combination of Microneedling and Chemical Peels

In recent years, a combination of chemical peels and antioxidants has become an option to rejuvenate the skin without any damage. Microneedle mesotherapy can increase the penetration of active ingredients. According to the reference (Wadstein *et al.*, 2022), the entire face was first treated with azelaic acid, and next, a 40% vitamin C solution was administered to the right side and 10% vitamin C to the left side using microneedling. Some skin characteristics like skin hydration and elasticity were significantly improved, and better results

were observed with microneedling. Melanin and erythema index decreased with treatment. No serious side effects were observed. The combination of active ingredients and delivery technologies has great potential to enhance the efficacy of cosmetic formulations, possibly through multidirectional mechanisms of action. In that study, both 20% azelaic acid + 40% vitamin C treatment and 20% azelaic acid + 10% vitamin C + microneedle mesotherapy treatment were effective in improving skin aging parameter evaluation. They have proven that the use of microneedling and mesotherapy techniques serves as a means of delivering active ingredients directly to the dermis and improves the efficacy of the studied formulations. Skin aging is a complex and multifactorial process that appears gradually from the third decade of life and accelerates with age (Wadstein *et al.*, 2022). As aging occurs earlier than in other tissues, likely due to environmental stressors and exposure to hazards, the main characteristics of skin aging commonly include loss of elasticity, appearance of wrinkles, and a rough texture (Zhang and Duan, 2018). These changes are associated with phenotypic changes in skin cells and structural and functional changes in extracellular matrix components such as elastin, collagen, and proteoglycans. These components are necessary for skin elasticity, tensile strength, and hydration (Huertas *et al.*, 2016). Skin aging is reflected by a decrease in collagen I content, fragmentation of collagen fibrils, and accumulation of amorphous elastin material (elastosis) (Aldag *et al.*, 2016). Age-related changes in skin appearance can negatively impact self-esteem and lead to significant psychosocial stress (Wadstein *et al.*, 2022). Therefore, products that help slow down this ongoing process are very important in daily life. A wide range of cosmetic and medical anti-aging products have been developed and introduced into the market. However, their efficiency varies. Despite manufacturers' guarantees, only a few topical anti-aging ingredients can effectively penetrate the dermis. Thus, various resurfacing techniques such as chemical peels, mesotherapy, filler injections, and laser/radio-frequency tightening are used to improve the delivery of active substances. The results show that all skin parameters evaluated were significantly improved after both treatment types. Both 20% azelaic acid + 40% vitamin C treatment and 20% azelaic acid + 10% vitamin C + microneedling mesotherapy effectively improved skin hyperpigmentation and erythema. Other related studies have also shown that azelaic acid has a positive impact on hyperpigmentation by inhibiting tyrosinase and exerting antiproliferative effects on the melanin production pathway (Bergman and Luke, 2017; Nautiyal and Wairkar, 2021). So, a combination of 5–20% azelaic acid and phytic acid, ferulic acid, 4N-butylresorcinol, or mandelic acid is most effective in reducing hyperpigmentation. Additionally, the use of vitamin C has been found to successfully reduce pig-

mentation (Mazurek and Pierzchała, 2016). A clinical trial evaluating the efficacy of a formulation containing 25% L-ascorbic acid and a chemical penetration enhancer demonstrated a significant reduction in pigmentation severity in patients with melasma (Hwang *et al.*, 2009). Vitamin C has been shown to affect blood microcirculation and reduce skin redness. Furthermore, the addition of ferulic acid not only increases the stability of vitamin C supplements but also improves whitening properties, as ferulic acid inhibits tyrosinase activity. Kameyama *et al.* (Oresajo *et al.*, 2008; Murray *et al.*, 2008) noticed that when MAP was used as a topical application to the skin, it actually bleached the skin in patients with hyperpigmentation diseases such as melasma and solar lentigines (Kameyama *et al.*, 1996). In this study, formal treatment provided patients with significant benefits in enhancing skin hydration, as well as in self-administered treatment (Algiert-Zielińska *et al.*, 2019). However, according to recent suggestions, this procedure may make the skin more moisturized (Matthews-Brzozowska *et al.*, 2017). In another study, azelaic acid was shown to balance sebum excretion and minimize aging effects, particularly dryness. Most conditions promote the skin to synthesize more collagen and elastin (Jung *et al.*, 2018; Tedeschi *et al.*, 2015). Fibroblasts synthesize various substances that help retain youthful skin condition (Jung *et al.*, 2018). The reason for this result is the use of active ingredient microinjection, which has been shown to stimulate the production of elastic/tendon and collagen fibers, promoting skin regeneration and repair during the healing process (Matthews-Brzozowska *et al.*, 2017). So, it seems that the enhanced skin elasticity achieved by the treatments—especially mesotherapy with microneedling—is not only due to vitamins but also the method of administration (Wójcik *et al.*, 2013).

18. Microneedling and Scar Treatment

The benefits of needles in scar treatment were first described over 20 years ago. Microneedling (MN), or percutaneous collagen induction therapy (PCI), has recently gained popularity due to its effectiveness in skin rejuvenation, retardation reduction, acne vulgaris, alopecia, scar remodeling, melasma, and other pigmentary diseases. Minimally invasive procedures use instruments with up to 540 needles that penetrate the epidermis and/or dermis to create microscopic channels (Yadav and Dogra, 2016). These needles have a diameter of 0.1 to 0.25 mm and a length of 0.5 to 3 mm. The small size of the needle allows drugs to penetrate into the dermis, and this controlled skin damage allows for the release of growth factors such as transforming growth factors alpha and beta (TGF α , TGF β) and fibroblast growth factor (FGF) (Yadav and Dogra,

2016). Ultimately, the microtrauma promotes the formation of new components like collagen, elastin, capillaries, and other skin substances. MN is a highly effective method that is usually used with various topical agents or other techniques to enhance the therapeutic efficacy of the topical agent or procedure itself. Generally, MN is a safe procedure, but sometimes unexpected side effects and reactions may occur. The literature used for this study was selected through a PubMed database search and matched with other topical reviews. The search terms included combinations of the following words: "Microneedling", "transdermal collagen induction", "collagen induction", "dermal roller", "dermal needling", "ring", "dermal rolling", "skin needling", "side effects", and "reaction". Adverse events were categorized based on the modality of MN studied: roller MN device (RMN), derma-stamp, pen MN device (PNM), and fractional radio-frequency MN device (RFMN). All reactions that occurred after combined treatment with MN were divided into appropriate sessions (Garg and Baveja, 2014; Majid, 2009; Sharad, 2011b; Leheta *et al.*, 2011; Khater *et al.*, 2016; Pahwa *et al.*, 2012). However, in one case with RMN, ecchymosis developed and resolved spontaneously within a few days (Park *et al.*, 2012). Ecchymosis may also be seen in RMN in patients without a history of bleeding disorders (Fabbrocini *et al.*, 2014; Dogra *et al.*, 2014). In referenced studies, 3 of 60 patients with subsequent 3 monthly RMN sessions—who did not have bleeding or collagen disease or were not receiving anticoagulant therapy—developed bony prominences. A mild ecchymotic lesion developed with no residual pigmentation. Other side effects have also been reported. Although rare, RMN use has been associated with these side effects (Fabbrocini *et al.*, 2014). It also caused scars (Dogra *et al.*, 2014; Pahwa *et al.*, 2012), milia (Sharad, 2011a; Asif *et al.*, 2016), Pruritus and eschar formations were recognized by RMN (Majid, 2009; Asif *et al.*, 2016; Cachafeiro *et al.*, 2016). In the case of acne scars in patients treated with RMN, there is a possibility of recurrence of acne scars (Leheta *et al.*, 2011; Asif *et al.*, 2016).

19. Effects of Microneedling on Acne and Melasma

In treating acne and melasma, due to its strong antibacterial and anti-inflammatory properties, microneedling has been successfully used in anti-acne treatments, melasma, and rosacea (Wójcik *et al.*, 2013). Before the treatment, the entire face was generally cleansed, and the skin was carefully cleaned with a pre-peel product containing 2% salicylic acid. Next, 20% azelaic acid (pH 2.7) was applied to the entire face for 10 minutes, and then the first steps of the main treatment were completed.



Fig. 4. This figure presents a representative case with multiple skin scars before treatment by the author using the microneedling method as part of a personalized skin rejuvenation protocol.

20. Microneedling Results

Microneedling results in the reorganization of old collagen fibers and the formation of new collagen, elastin, and capillaries, resulting in skin tightening. In this process, El-Domyati *et al.* showed that after six microneedling sessions every two weeks, levels of collagen types I, III, VII, newly synthesized collagen, and tropoelastin were significantly increased from baseline. According to references, this transdermal collagen in-

duction reduces fine lines and wrinkles, decreases pore size, and makes the skin more elastic and supple. As a result, it makes the skin more youthful and is a powerful function for skin rejuvenation. Combining this procedure with topical anti-aging vitamin C serum and tretinoin will further increase its effectiveness. Microneedling also demonstrated a significant reduction in wrinkles and pigmentation when combined with endothelial progenitor cells derived from human embryonic stem cells (Lee *et al.*, 2014; Seo *et al.*, 2013). In addition, radio-frequency fractionated microneedling was evaluated in a large multi-center study and found to be effective in reducing wrinkles (Calderhead *et al.*, 2013).



Fig. 5. This figure presents a representative case treated by the author after one session of microneedling as part of a personalized skin rejuvenation protocol. The intervention was conducted under clinical supervision, and the image demonstrates the observed therapeutic response.



Fig. 6. In pictures (A, B), on day 3, excess melanin pigments migrate toward the superficial part of the skin tissue. Damaged cells with excess pigments separate from the skin by day 4. On days 30, 60, and 90, the skin appears brighter, and rejuvenation is visible.



Fig. 7. Compared to before, the picture of the case on day 0 and after microneedling on day 120 shows visible effects on scars and melasma, skin lifting, stratum corneum debris removal, and overall skin rejuvenation.

21. After Facial Microneedling

A skin care protocol was developed for use after facial radio-frequency microneedling procedures. When used immediately after the procedure, it improves normal activity, comfort, perceived age, overall treatment satisfaction, and the need for re-treatment, while providing essential mineral sun protection factor, HEV protection, and defense against environmental pollution. This skin care protocol is also suitable for use after other minimally invasive facial treatments (Juhasz and Cohen, 2020). Most subjects (90%) were very likely to undergo another microneedling procedure after receiving the skin care protocol, and all subjects (100%) said their protocol was radio-frequency based and reported that microneedling influenced their perception of improvement. After this experience, 86% of participants felt very comfortable scheduling their next microneedling treatment on any day or time. Overall, 96% of subjects were very satisfied with their experience, making it a positive report (Iriarte *et al.*, 2017; Wu *et al.*, 2020). After the procedure, self-confidence and social life may be negatively affected. After applying post-procedure skin care products, subjects were shown before-and-after photos and asked to answer several questions. 50% of subjects felt very uncomfortable immediately after the procedure, whereas 94% would have otherwise felt very or extremely self-conscious in public. Additionally, 88% said their skin felt more comfortable after using skincare products, 95% felt less self-conscious in public, and 94% felt bet-

ter about their appearance after using the products. 97% said they intended to continue treatment and were more likely to recommend it to others. 87% felt their overall impression of the treatment process had improved. After 4 weeks, most participants felt their skin looked younger and healthier (94%). They were confident in repeating the treatment (94%) and continued using skin care products as part of their daily routine (94%). In these studies, the average age of subjects was 42.7 years (range: 23–63 years). Subjects were Caucasian, Fitzpatrick skin types I, II, III, and IV. Before applying the skin care protocol on day 1 post-treatment, a third group used a non-drug barrier ointment without any injection. Before treatment, more than half (59%) of participants reported being very concerned about their appearance. After all processes, 52% of subjects seemed unsure of their appearance following radio-frequency microneedling treatment (Iriarte *et al.*, 2017; Wu *et al.*, 2020; Kim *et al.*, 2013).

22. Lifestyle and Rejuvenation

In recent years, life expectancy and demand for youthful skin have increased, creating a demand for effective cosmetic facial treatments—especially non-invasive, non-surgical, and low-risk skin rejuvenation methods. Aging is associated with morphological changes in the skin, caused by a decrease in the number of fibroblasts and levels of collagen, elastin, and glycosaminoglycan (GAG) biosynthesis.



Fig. 8. A comparative evaluation of the patient's skin condition between the first session on day 3 and the fourth session on day 120 after microneedling revealed substantial improvements in dermal texture, pigmentation uniformity, and overall skin vitality. The observed changes suggest that microneedling induces not only superficial enhancement but also deep-level rejuvenation through stimulation of neocollagenesis and dermal remodeling. The degree of transformation—evident in both clinical photographs and tactile assessment—highlights microneedling as a highly effective and non-invasive modality for skin rejuvenation. The progressive reduction in hyperpigmentation, smoothing of fine lines, and restoration of epidermal tone collectively support its role as a superior therapeutic approach in aesthetic dermatology.

These changes can cause skin laxity, structural alterations, and wrinkles (Uitto, 2008). According to the aim of treatments, insulated or non-insulated microneedles are used to penetrate the skin, resulting in partial destruction of the epidermis and subsequent regeneration. The RF energy produced by the microneedle heats the superficial and deep dermis, creating zones of clinically imperceptible thermal micro-wounds between areas of normal, healthy skin, which trigger a wound healing cascade (Arnoczky and Aksan, 2000; Dierickx, 2006). Zones of normal and untreated skin improve skin texture, reduce wrinkles, and tighten the skin due to the formation of new collagen, elastin, and GAGs in response to treatment (Hantash *et al.*, 2009b; Hantash *et al.*, 2009a; Hruza *et al.*, 2009; Seo *et al.*, 2012; Lee *et al.*, 2011a; Gold *et al.*, 2016; Tanaka, 2015; Alexiades-Armenakas *et al.*, 2010; Shapiro, 2015). According to the results, the primary endpoint was met, and improvements in skin texture and wrinkles were clearly detected. The researchers assessed and showed statistically significant improvements one month after the sixth treatment session and three months after the

third session ($p < 0.005$ at both time points).

23. Clinical Study

According to references, in one clinical study, twenty-five participants were recruited for a prospective, randomized, controlled, observer-blinded, split-face study. Most participants were between 41 and 64 years old (mean: 51.6 years) and had Fitzpatrick skin type III or IV. Extrinsic senescence affects cellular and extracellular components. As an alternative treatment strategy for repairing damaged tissues, cell-based treatments using the body's own stem cells and growth factors have recently been applied to skin rejuvenation. In addition to their direct cellular effects, stem cells exert beneficial effects on tissue regeneration through complex paracrine mechanisms (Cha and Falanga, 2007). Previous studies found that hESC-EPC conditioned media (CMs) promoted wound healing and increased wound tensile strength after topical treatment and subcutaneous injection. In vitro, hESC-EPC CM significantly improved mechanisms such as proliferation and migra-

tion of dermal fibroblasts, epidermal keratinocytes, and collagen synthesis (Lee *et al.*, 2011b). Additionally, conditioned medium from adipose tissue-derived stem cells (ADSC CM) inhibited melanogenesis by down-regulating the expression of tyrosinase and tyrosinase-related protein 1 in B16 melanoma cells and ADSCs (Kim *et al.*, 2009b). It was hypothesized that hESC-EPC CM can improve signs of skin aging such as wrinkles and pigmentation. This result highlights the importance of transdermal penetration and epidermal-dermal communication for the application of growth factors in skin rejuvenation. In another study, 48 subjects were selected and all completed treatment. No subjects discontinued due to protocol-related problems or adverse events. The mean age was 55.1 years (range: 39–67 years), and the subjects were primarily female. Fitzpatrick phototype ranged from I to V, with an average of 2.2. Baseline scores for wrinkles, skin laxity, and skin texture were 3.2 (range: 2.2–4), 2.8 (2–3.5), and 2.7 (1.5–3.5), respectively. Changes were summarized at days 90 and 150. At day 90, four facial areas improved at least one level: glabellar wrinkles, periorbital wrinkles, cheek wrinkles, and lip wrinkles. The mean improvement in total score was 0.93 (99%). By day 150, six of nine facial areas improved at least one level: horizontal forehead lines, glabellar lines, periorbital lines, cheek lines, upper lip lines, and lip lines. The mean global score improvement was 1.23 (99%) with $p = 2.5 \times 10^6$ (i.e., highly significant). Average improvements were observed in all roughness parameters in the periorbital and mid-labial regions at both 90 and 150 days. Overall, microneedling or PCI appears to be effective and successful in treating facial wrinkles, sagging skin, and skin texture issues. Previous studies lacked sufficient sample sizes. In the present study, a microneedle system was evaluated for treating facial aging in a moderately sized patient sample (Cha and Falanga, 2007; Lee *et al.*, 2011b; Kim *et al.*, 2009b).

24. Discussion

The recent microneedling method is one of the most effective treatments for achieving healthy and youthful skin texture and rejuvenation. This method is especially beneficial for patients with Fitzpatrick skin types IV and V. It is a powerful technique that can resolve scar problems and hyperpigmentation successfully. Unlike other methods that may have side effects, microneedling is safer and has numerous applications with a growing number of modifications. This method is considered a valuable technology. We do not recommend self-use of dermapen or microneedling devices. It is best to receive treatment from a specialized doctor. The results of endogenous aging include clinical symptoms such as genetic mutations, hormonal changes, and metabolic shifts, while extrinsic factors include pol-

lutants, ultraviolet radiation, and ionizing radiation (Makrantonaki and Zouboulis, 2007). Over time, aging and photoaging due to environmental factors lead to changes in pigmentation, loss of elasticity, fine lines and wrinkles, moisture loss, and telangiectasia—thus increasing the risk of skin cancer (Mukherjee *et al.*, 2011). Additionally, aged skin undergoes extracellular matrix atrophy and destruction of collagen and elastic fibers, manifesting as epidermal thinning and flattening of the dermal-epidermal junction (Makrantonaki and Zouboulis, 2007). Microneedling is one of the best ways to prevent these negative changes in skin texture. Photoaged skin is characterized by the accumulation of abnormal elastic tissue called solar elastosis. This abnormal tissue can replace the normal matrix, which is primarily composed of collagen. Glycosaminoglycans are usually distributed between collagen bundles and have the ability to bind water, increasing skin hydration. Although they increase in photoaged skin, their function is altered due to association with abnormal elastic fibers (Makrantonaki and Zouboulis, 2007). Collagen is the most important component of human skin, synthesized from procollagen derived from dermal fibroblasts. Transforming growth factor β (TGF- β) is a cytokine that regulates dermal fibroblasts and may promote collagen degradation via upregulation of matrix metalloproteinases (MMPs). Extrinsic factors can promote reactive oxygen species (ROS), increasing MMP-mediated collagen degradation and inflammation through the NF- κ B pathway. Ultraviolet (UV) light can directly cause DNA cross-linking and modification of structural proteins (Mukherjee *et al.*, 2011; Aldag *et al.*, 2016).

Therefore, the effects of the topical application of growth factors have been studied for skin remodeling and wound healing. In this process, there are other proteins in the skin which are considered of great importance—these are growth factors. These are proteins produced by cells such as keratinocytes, fibrocytes, and melanocytes, and they affect collagen synthesis. These cytokines include platelet-derived growth factor, epidermal growth factor (EGF), granulocyte colony-stimulating factor, vascular endothelial growth factor (VEGF), keratinocyte growth factor, and hepatocyte growth factor. Since growth factors cannot pass through skin layers, their molecular weights restrict them—most are greater than 15,000 kDa. In fact, molecules greater than 500 kDa cannot penetrate the stratum corneum. However, when microneedles are used to pierce the skin at different layers, large molecules may penetrate more easily. These molecules include cytokines, platelet-derived growth factor, EGF, granulocyte colony-stimulating factor, VEGF, keratinocyte growth factor, and hepatocyte growth factor (Aldag *et al.*, 2016; Sproul and Argraves, 2013; Uitto and Kouba, 2000; Verrecchia and Mauviel, 2007). Due to their molecular size, growth

factors cannot enter the stratum corneum unless an entry route such as microneedling is provided. Stem cell biology and its applications in tissue engineering and regeneration involve three primary properties, including cell migration. Migration produces intercellular matrix, and fibroblast proliferation leads to collagen fiber deposition (Aldag *et al.*, 2016). The effect of skin tightening and collagen augmentation remains for 5 to 7 years after the procedure (Schaefer and Lademann, 2001). In the current research, the outcomes of microneedling in combination were compared with a recently introduced topically applied growth factor. These variables are believed to reduce inflammation recovery time and progressively enhance textural character by stimulating collagen synthesis induced by microneedling.

As the Future Perspective, in a split-face clinical study, the effectiveness of the microneedle patch in improving nasolabial folds was investigated. As evaluated by dermatologists, both the microneedle patch with HA solution and the microneedle patch alone significantly improved the Mertz aesthetic score of the nasolabial folds. Both patient groups showed significant improvement from week 4 to the end of the study, but no significant difference was observed between the two groups. Possibly, the low concentration (1.8%) of the HA solution was the reason for this result. In theory, both the microneedle patch and the 1.8% HA solution can improve wrinkles. However, the effect of the microneedle patch may be greater than applying 1.8% HA. According to data, there was no significant difference between the two groups in wrinkle improvement. Nonetheless, the satisfaction score for the microneedle patch with HA solution group was higher than the microneedle patch alone group, based on self-ratings by participants (Yaar *et al.*, 2002). This improvement may be due to the percutaneous microtrauma caused by the microneedle patch and increased permeability of the drug across the channels created by microneedles (Gilchrest, 1989). Many previous studies have suggested the utility of microneedle devices for delivering substances through the skin (Tammi *et al.*, 2005). Tammi *et al.* showed that skin microtrauma itself can stimulate epidermal HA synthesis via upregulation of hyaluronan synthase expression. In dermatology, medical needling causes skin color normalization and healthy skin adaptation after repeated treatments of hypertrophic burn scars (Kay-Hendrik *et al.*, 2018). Kim *et al.* showed that in cosmetics, due to their patient-friendliness, safety, and effectiveness in improving wrinkles, microneedle patches can be efficiently and confidently used in various conditions (Kim *et al.*, 2014). Microtrauma with small needles and subsequent tissue regeneration can induce collagen synthesis and deposition, resulting in skin rejuvenation and improvement. Furthermore, the needle-stick device itself has been shown to influence angiogenesis by stimulating

it in the post-needle wound healing cascade. Specifically, epidermal penetration causes microtrauma and intradermal hemorrhage through parenchymal channels without affecting the basal layer's regenerative ability of stem cells. In this way, transdermal collagen induction enhances the endogenous potential for regeneration, and a modified wound healing cascade increases the expression of growth factors such as VEGF (Bao *et al.*, 2009) and TGF- β (Faler *et al.*, 2006), both of which are important in angiogenesis and cell differentiation. Medical needles stimulate both gene expression and skin cell proliferation, which are essential for skin remodeling. Transdermal collagen induction can alter the TGF- β signaling pathway, as TGF- β 3 reaches high expression levels during early wound healing stages. This factor allows scar-free collagen synthesis and wound healing (Bandyopadhyay *et al.*, 2006; Ferguson and O'Kane, 2004). Moreover, the post-needling cascade leads to the formation of a physiological lattice-like collagen matrix of type I rather than type III collagen, which is parallel-oriented and less strong and stable (Aust *et al.*, 2008a). Over the past decade, radio-frequency microneedling (MRF) has been widely used to combat skin aging and is a useful method for treating various skin problems (Tan *et al.*, 2021; Dayan *et al.*, 2019; Alessa and Bloom, 2020).

25. Conclusion

Microneedle patch alone has the same effect as adding 1.8% HA solution to the microneedle patch for improving nasolabial folds. Using the microneedle patch alone is expected to improve wrinkles due to its favorable safety profile. Because the current study focused only on female participants and a low concentration of non-cross-linked HA solution, it is possible to apply this to a larger population, a wider range of skin types, and higher concentrations—or perhaps a cross-linked HA solution. A clinical trial is scheduled to be conducted. Linked HA solutions are beneficial in extending these results. Additionally, long-term follow-up is needed to examine the persistence of the observed effects. Future clinical evaluation of the technology may support previous authors' suggestions that microneedling is the most reliable alternative to more ablative therapies, particularly in skin phototypes IV to V. Such studies would benefit from research groups that include more ethnically diverse populations. This technique does not cause thermal or excessive epidermal damage. Microneedling is a simple procedure that can be performed in-office. The device is cost-effective, and the only recurring cost is the disposable microneedle cartridge. The treatment appears to be well tolerated, with minimal pain, discomfort, and downtime. Compared to other invasive techniques such as laser ablation and radio-frequency, side effects appear to be mi-

nor and easily managed. Therefore, we suggest choosing this method to guarantee skin health and restore youthful skin.

Author Contributions

All processes for preparing the draft, writing, and editing were done by Arash Younesi as the first author. Under the supervision of Professor Hassan Morovvati and Hossein Najafzadeh Varzi, they have read, reviewed, and agreed to the published version of the manuscript.

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ORIGINAL RESEARCH PAPER

ABCB1 Promoter Methylation: A Potential Biomarker and Therapeutic Target in the Pathogenesis of Ulcerative Colitis

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Abstract

The aim of this systematic review was to synthesize the existing evidence on the role of ABCB1 promoter methylation in the pathogenesis of ulcerative colitis (UC) and to evaluate its potential as a biomarker and therapeutic target. A systematic search was conducted across PubMed/MEDLINE, Scopus, Embase, Web of Science, and the Cochrane Library for articles published up to March 2024. Original human, animal, and cellular studies examining the association between ABCB1 methylation and UC were selected based on predetermined criteria, and their quality was assessed using standard tools. A total of 38 studies met the inclusion criteria. The evidence consistently indicated that ABCB1 promoter methylation levels in the colonic tissue of UC patients were significantly higher than in healthy individuals. A significant inverse correlation between increased methylation levels and decreased expression of ABCB1 mRNA and P-glycoprotein was reported in most studies. Furthermore, ABCB1 hypermethylation was associated with increased disease severity, a higher risk of relapse, and a poorer response to standard treatments. Laboratory studies demonstrated that pharmacological interventions and natural compounds could reverse this epigenetic alteration. Therefore, it can be concluded that ABCB1 promoter hypermethylation is an important epigenetic event in UC pathogenesis, contributing to intestinal barrier dysfunction and inflammation by silencing a key protective gene. This marker has high potential for application in the diagnosis, prognosis, and personalized treatment of UC.

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohns disease, are chronic and debilitating disorders characterized by gastrointestinal inflammation. The global prevalence of IBD, particularly UC, has dramatically increased in recent decades (Kaplan & Ng, 2017; Ng *et al.*, 2018). UC pathogenesis is a complex multifactorial process arising from interac-

tions between genetic susceptibility, inappropriate immune responses, environmental factors, and gut microbiota (Ungaro *et al.*, 2017; Danese and Fiocchi, 2019). Alongside genetic factors, growing evidence emphasizes the vital role of epigenetic changes, particularly DNA methylation, in UC pathogenesis (Kellermayer, 2012). DNA methylation is a reversible chemical modification where a methyl group is added to cytosines, primarily

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in CpG contexts. Hypermethylation (increased methylation) in gene promoter regions typically leads to altered chromatin structure and transcriptional silencing of target genes (Jones, 2012). Numerous studies have identified abnormal methylation patterns in colonic tissue of UC patients associated with disruption of key pathways such as intestinal barrier function and inflammatory responses (Ventham *et al.*, 2013; Häslér *et al.*, 2012). One of the key genes strongly affected by these epigenetic c Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn’s disease, are chronic and debilitating disorders characterized by gastrointestinal inflammation. The global prevalence of IBD, particularly UC, has dramatically increased in recent decades (Kaplan changes in UC is ABCB1 (also known as MDR1). This gene encodes an important membrane protein called P-glycoprotein (P-gp). P-gp is an ATP-dependent transport pump that plays a vital role in maintaining mucosal barrier integrity by actively pumping xenobiotics, toxins, and inflammatory metabolites out of intestinal epithelial cells (Ambudkar *et al.*, 2003; Gottesman *et al.*, 1996; Thiebaut *et al.*, 1987). Downregulation of P-gp expression or function can lead to accumulation of harmful substances within the cells, increasing the intestinal permeability, and exacerbation of inflammatory responses (Blokzijl *et al.*, 2007; Langmann *et al.*, 2004). Initial evidence has shown that ABCB1 expression is reduced in inflamed mucosa of UC patients, and promoter hypermethylation has been proposed as the main mechanism for this phenomenon (Tahara *et al.*, 2009; Gazouli *et al.*, 2010). This hypothesis is strengthened when Abcb1a knockout mice (the mouse homolog of human ABCB1) are observed to develop severe colitis spontaneously similar to human UC (Panwala *et al.*, 1998). While numerous individual studies have highlighted a link between ABCB1 methylation and UC, a systematic synthesis evaluating the consistency and strength of this evidence across diverse populations, methodologies, and clinical endpoints is currently lacking. Key questions remain unresolved: How consistent is the finding of ABCB1 hypermethylation in UC? Does this epigenetic mark hold potential as a reliable biomarker for predicting disease severity, relapse, or treatment response? The absence of a consolidated overview limits its clinical translation. Therefore, this systematic review aims to address this gap by synthesizing all available evidence to provide a comprehensive understanding of the role of ABCB1 promoter methylation in UC pathogenesis and to critically evaluate its potential as a clinical biomarker and therapeutic target.

Methods

1.1. Protocol and Registration

This systematic review was designed and reported according to the guidelines of the “Preferred Report-

ing Items for Systematic Reviews and Meta-Analyses” (PRISMA 2020) statement (Page *et al.*, 2021). The protocol for this systematic review was designed prior to commencement and registered in the International Prospective Register of Systematic Reviews (PROSPERO), with registration number pending.

1.2. Search Strategy

The search strategy was formulated by combining keywords related to the disease (Ulcerative Colitis, IBD), the gene of interest (ABCB1, MDR1, P-glycoprotein), and the epigenetic process (DNA methylation, hypermethylation, epigenetics) using Boolean operators (AND, OR).

1.3. Inclusion and Exclusion Criteria

Studies for inclusion in this review had to meet the following criteria: study type (original), population (UC patients, animal or cellular models), exposure (assessment of ABCB1 methylation), and presence of a comparison group. Review articles and studies without a control group were excluded.

1.4. Study Selection, Data Extraction, and Quality Assessment

After removing duplicates, two independent researchers reviewed titles and abstracts, followed by full-text articles. Data were extracted using a standardized form. The quality of human studies was assessed using the Newcastle-Ottawa Scale (NOS) (Wells *et al.*) and animal studies with SYRCLE’s risk of bias tool (Hooijmans *et al.*, 2014). Due to heterogeneity, results were presented as a qualitative synthesis. A quantitative meta-analysis was deemed inappropriate due to the significant heterogeneity across studies in terms of methylation assessment methodologies (e.g., MSP, Pyrosequencing, arrays), patient populations, and reported outcome measures. Therefore, a structured narrative synthesis was performed.

1.5. Data Synthesis

We performed a thematic synthesis of the findings. Key data related to the association between ABCB1 methylation and UC, clinical outcomes, and gene expression were extracted from each study. These findings were then grouped into overarching themes. We analyzed the consistency and discrepancies of findings within each theme to build a comprehensive narrative of the current evidence.

2. Results

2.1. Study Selection

The initial search identified 127 articles. After removing duplicates and screening based on title, abstract, and full text, 36 studies (including 23 human studies, 9 animal studies, and 4 cellular studies) were ultimately selected for inclusion in this systematic review.

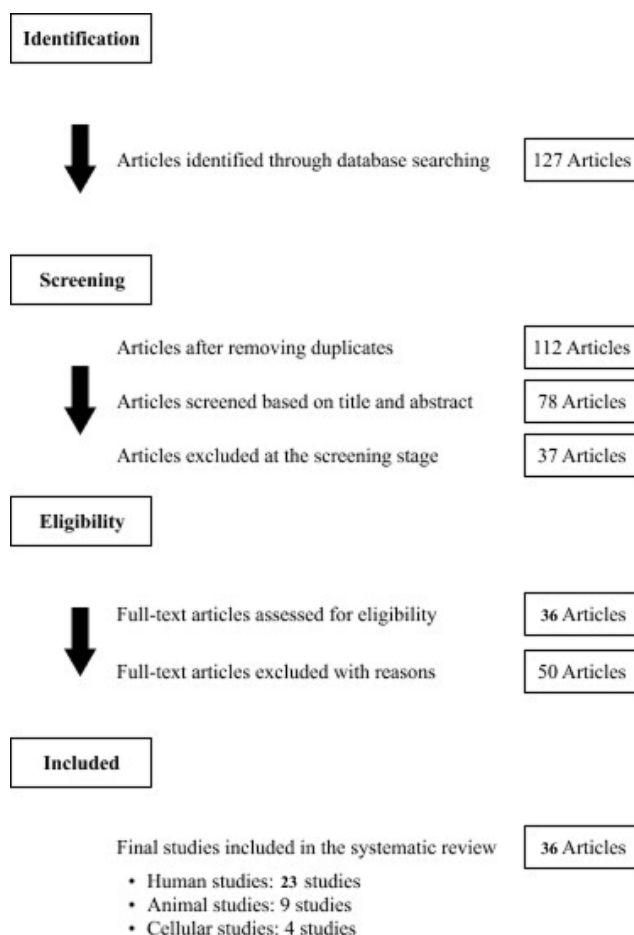


Fig. 1. PRISMA flow diagram showing the study selection process (Page *et al.*, 2021).

2.2. Qualitative Synthesis of Findings

2.3. ABCB1 Promoter Hypermethylation is a Consistent Finding in UC Colonic Tissue

The most consistent finding across the included human studies was the significant hypermethylation of the ABCB1 promoter in the colonic tissue of UC patients compared to healthy controls. This observation was robust, holding true across various geographical populations (e.g., Japan, Iran, Greece) and diverse measurement techniques, from qualitative MSP (Sadeghi *et al.*, 2020; Gazouli *et al.*, 2010) to quantitative pyrosequencing (Tahara *et al.*, 2009) and genome-wide arrays (Häsler *et al.*, 2012). This strong consistency suggests

that ABCB1 hypermethylation is a fundamental epigenetic feature of the disease in intestinal tissue.

2.4. Functional Consequence: Silencing of Gene Expression

The functional impact of this hypermethylation was clearly demonstrated by an inverse correlation with gene expression. The majority of studies that assessed both methylation and expression levels reported that increased promoter methylation was significantly associated with decreased ABCB1 mRNA and P-glycoprotein levels (Blokzijl *et al.*, 2007; Novak *et al.*, 2022). This provides a mechanistic link between the epigenetic alteration and the loss of the protective P-gp pump function, a key element in UC pathogenesis.

3. Discussion

This systematic review synthesizes compelling evidence establishing that ABCB1 promoter hypermethylation is a central and consistent epigenetic event in the pathogenesis of ulcerative colitis (UC). The key findings demonstrate that: (1) ABCB1 methylation levels are significantly elevated in the colonic tissue of UC patients compared to healthy controls; (2) this hypermethylation functionally leads to transcriptional silencing and reduced expression of the key P-glycoprotein (P-gp) transporter; and (3) methylation levels correlate directly with disease severity, prognosis, and therapeutic response. Collectively, these findings provide a cohesive framework for a critical pathogenic mechanism in UC. The mechanistic interpretation of these findings is rooted in the vital role of P-gp as an efflux pump in intestinal epithelial cells. This transporter actively expels xenobiotics, bacterial toxins, and inflammatory metabolites, forming an integral part of the intestinal barrier. Epigenetic silencing of the ABCB1 gene via hypermethylation compromises this defensive barrier, leading to intracellular accumulation of noxious substances, increased intestinal permeability, and an exacerbated inflammatory response. This concept is strongly supported by animal models, where *Abcb1a* knockout mice spontaneously develop a severe colitis that closely mimics human UC (Panwala *et al.*, 1998), confirming a causal link between the loss of P-gp function and intestinal inflammation. Beyond its mechanistic role, these findings have profound clinical implications, highlighting ABCB1 methylation as a potential multifaceted biomarker. Prognostic and Diagnostic Potential: The consistent correlation with disease severity (Matsumura *et al.*, 2019) and a higher risk of relapse in patients in remission (Müller *et al.*, 2020; Cohen *et al.*, 2024) positions it as a powerful prognostic tool. Critically, ABCB1 hypermethylation has also been identified as a risk factor for the development of colitis-associated cancer (Tahara *et al.*, 2014).

Table 1

Characteristics of selected studies included in the systematic review.

No.	Author (Year)	Study type / country	Population / Sample	Methylation assessment method	Key findings
1	Tahara <i>et al.</i> (2009)	Case-control / Japan	85 UC patients, 90 healthy controls (colon biopsy)	Quantitative	Significant increase in methylation in UC patients ($p < 0.001$) and its association with disease activity.
2	Sadeghi <i>et al.</i> (2020)	Case-control / Iran	50 UC patients, 50 controls (colon biopsy)	MSP	High frequency of hypermethylation in UC patients (78%) compared to control group (12%).
3	Gazouli <i>et al.</i> (2010)	Case-control / Greece	98 IBD patients, 52 controls (colon biopsy)	MSP	High prevalence of PGP promoter hypermethylation in patients (61.2%) compared to controls (15.4%).
5	Schmidt <i>et al.</i> (2018)	Cohort / Germany	120 UC patients (peripheral blood - PBMCs)	Quantitative MSP	High methylation in blood was predictive of poor response to glucocorticoid treatment.
6	Matsumura <i>et al.</i> (2019)	Case-control / Japan	102 UC patients, 40 controls (colon biopsy)	Quantitative pyrosequencing	Methylation had positive correlation with endoscopic indices of disease severity.
7	Tahara <i>et al.</i> (2014)	Case-control / Japan	185 UC patients (colon biopsy)	Quantitative pyrosequencing	ABCB1 methylation was identified as a risk factor for colitis-associated cancer development.
8	Cui G, <i>et al.</i> (2016)	Case-control / China	76 UC patients, 30 controls (colon tissue)	MSP	ABCB1 hypermethylation was associated with decreased expression
9	He H, <i>et al.</i> (2015)	Case-control / China	82 UC patients, 45 controls (colon tissue)	MSP	ABCB1 hypermethylation was associated with decreased expression
10	Gholam-Rezaei <i>et al.</i> (2018)	Case-control / Iran	100 IBD patients, 100 controls (peripheral blood)	ARMS-PCR	Significant association between MDR1 promoter hypermethylation and susceptibility to UC.
11	Kanaan Z, <i>et al.</i> (2013)	Case-control / USA	36 UC patients, 18 controls (colon biopsy)	Array-based	No significant association was observed between ABCB1 promoter methylation and IBD risk (contradictory results).
12	Häsler R, <i>et al.</i> (2012)	Case-control / Germany	22 UC patients, 13 controls (colon biopsy)	Genome-wide BS-Seq	ABCB1 was one of the genes with abnormal hypermethylation in UC patients.
13	Gassner C, <i>et al.</i> (2016)	Review- / analytical Germany	-	-	UC methylome map showed ABCB1 as one of the differentially methylated regions.
14	Blokzijl H, <i>et al.</i> (2007)	Observational / Netherlands	15 UC patients, 10 controls (colon biopsy)	IHC, Real-time PCR	Analysis of evidence regarding the role of epigenetic regulation of ABCB1 in IBD pathology.
15	Langmann T, <i>et al.</i> (2004)	Observational / Germany	12 UC patients, 10 controls (colon biopsy)	Microarray	Decreased expression of P-gp/MDR1 protein and mRNA in inflamed intestinal tissue was confirmed.
16	Panwala <i>et al.</i> (1998)	Animal / USA	mdr1a knockout mice (colon tissue)	Histology	Significant decrease in expression of metabolism-related genes (including ABCB1) in UC.
17	Chen <i>et al.</i> (2019)	Animal / China	DSS colitis model mice (colon tissue)	Bisulfite sequencing	Knockout mice spontaneously developed severe colitis similar to human IBD.
18	Liu <i>et al.</i> (2020)	Animal and cellular / China	DSS colitis model mice, HT-29 cells	Western Blot, Western Blot, PCR	Resveratrol was able to reverse Abcb1 promoter hypermethylation and improve inflammation.
19	Ikeda <i>et al.</i> (2018)	Laboratory / Japan	Caco-2 cell line	Pyrosequencing, WB	Curcumin increased P-gp expression in colitis and cellular models.
20	Schwab M, <i>et al.</i> (2007)	Animal / Germany	DSS and TNBS colitis model mice	Real-time PCR	Mesalamine (5-ASA) reduced methylation and increased P-gp expression through DNMT inhibition.
21	Martin R, <i>et al.</i> (2021)	Laboratory / France	T84 cell line, co-culture with E. coli bacteria	COBRA, Real-time PCR	Activation of PXR (upstream regulator of ABCB1) improves colitis.
22	Wang L, <i>et al.</i> (2022)	Animal / China	TNBS colitis model mice	MeDIP-Seq	Exposure to pathogenic E. coli strains increased DNMT1 expression and ABCB1 hypermethylation.
					Fiber-rich diet (butyrate) prevented ABCB1 hypermethylation and reduced inflammation.

No.	Author (Year)	Study type / country	Population / Sample	Methylation assessment method	Key findings
23	Jensen P, <i>et al.</i> (2020)	Laboratory / Denmark	Human colon organoids derived from UC patients	Pyrosequencing, WB P	TNF- α treatment directly reduced P-gp expression and increased its promoter methylation.
24	Fischer A, <i>et al.</i> (2019)	Animal / Germany	IL-10 knockout mice	Bisulfite sequencing	These mice showed progressive ABCB1 hypermethylation from early age that correlated with colitis severity.
25	Patel S, <i>et al.</i> (2023)	Laboratory / USA	HT-29 cells, using siRNA	Real-, time PCR, WB	Knocking down DNMT1 enzyme with siRNA successfully restored ABCB1 expression.
26	Romano C, <i>et al.</i> (2019)	Case-control Italy	60 pediatric UC patients, 50 controls (rectal biopsy)	Quantitative MSP	Pediatric patients with early-onset disease showed higher methylation levels compared to adults.
27	O'Connell J, <i>et al.</i> (2021)	Cohort / Ireland Cohort / Ireland	95 UC patients undergoing Infliximab treatment (biopsy)	Pyrosequencing	Low ABCB1 methylation level before treatment was associated with better clinical response to Infliximab.
28	Lee B, <i>et al.</i> (2022)	Case-control / South Korea	250 IBD patients, 200 controls (peripheral blood)	MassARRAY	Gene-epigene interaction study: C3435T polymorphism was associated with increased risk of hypermethylation.
29	Müller K, <i>et al.</i> (2020)	Longitudinal / Switzerland	50 UC patients in remission (annual biopsies)	Quantitative MSP	In patients who relapsed, methylation levels were increased in samples before relapse.
30	Harris N, <i>et al.</i> (2023)	Case-control / Australia	140 UC patients, 80 controls (sigmoid and rectal biopsies)	Illumina EPIC Array	Comprehensive methylome analysis confirmed ABCB1 as one of the top 10 genes with hypermethylation in UC.
31	Silva A, <i>et al.</i> (2021)	Case-control / Brazil	70 UC patients, 60 controls (colon tissue)	MSP	Association between high methylation levels and need for higher mesalamine doses to control disease.
32	Petrov V, <i>et al.</i> (2022)	Case-control / Russia	88 UC patients, 75 controls (peripheral blood)	Real-time PCR & MSP	Correlation between increased hs-CRP levels (systemic inflammation marker) and ABCB1 methylation level in blood.
33	Cohen E, <i>et al.</i> (2024)	Cohort / Canada	210 UC patients (non-inflamed rectal tissue)	Pyrosequencing	High methylation in apparently healthy tissue was an independent risk factor for disease relapse within 2 years.
34	Al-Mansoori F, <i>et al.</i> (2023)	Case-control UAE	65 UC patients, 50 controls (colon biopsy)	MSP	Methylation level was significantly higher in patients with positive family history of IBD.
35	Novak J, <i>et al.</i> (2022)	Observational / Poland	110 UC patients (colon tissue)	IHC, MSP	Strong inverse correlation between number of P-gp+ cells in tissue and promoter methylation level was observed.
36	Garcia F, <i>et al.</i> (2021)	Case-control / Spain	90 UC patients, 80 controls (colon biopsy)	MSP	Association between hypermethylation and decreased intestinal microbiota diversity in UC patients.

Predictive Potential: This marker could be instrumental in personalizing therapy. Studies have shown that high pre-treatment methylation levels are associated with a poorer response to glucocorticoids (Schmidt et al., 2018) and the biologic agent infliximab (O'Connell et al., 2021). This information could guide clinicians in selecting more effective treatment strategies. The emergence of non-invasive assessment techniques, such as methylation analysis in circulating cell-free DNA (cfDNA), may bring these applications closer to routine clinical practice. One of the most compelling aspects of this finding is the inherent reversibility of epigenetic modifications, making ABCB1 a promising therapeutic target. In vitro and in vivo studies have demonstrated that natural compounds, such as resveratrol and curcumin, as well as common medications like mesalamine (5-ASA), can reverse ABCB1 hypermethylation and restore its expression (Chen et al., 2019; Ikeda et al., 2018). This opens an avenue for de-

veloping 'epigenetic therapies' that specifically target DNA methyltransferases (DNMTs) to reactivate this protective gene. However, it is important to address discrepant findings in the literature. For instance, the study by Gholam-Rezaei et al. (2018) found no significant association in peripheral blood samples. This discrepancy likely highlights the tissue-specific nature of epigenetic alterations in UC, where the primary pathological changes occur in the inflamed colonic tissue and may not be fully mirrored in the peripheral circulation.

4. Limitations

Despite its robust findings, this review has several limitations. First, significant methodological heterogeneity across the included studies, particularly in the variety of methylation assays (from qualitative MSP to quantitative pyrosequencing), complicates the direct

comparison of methylation levels. Second, the predominance of cross-sectional study designs prevents definitive conclusions about causality; more longitudinal studies are required to validate the prognostic role of this marker. Third, potential confounding factors, such as diet (Wang et al., 2022), medication use (especially mesalamine), and the genetic background of patients (e.g., the C3435T polymorphism), were not consistently controlled for in all studies. Finally, the possibility of publication bias, a tendency to publish positive results, may have influenced the overall observed strength of the association.

5. Conclusion

ABC1 promoter hypermethylation is a key epigenetic event in the pathogenesis of UC, holding significant potential as a diagnostic, prognostic, and predictive biomarker. Future research should focus on standardizing measurement protocols, conducting large-scale longitudinal cohort studies to validate its clinical utility, and exploring the therapeutic potential of epigenetic drugs. Translating this valuable knowledge into clinical practice could ultimately lead to improved management and outcomes for patients with UC.

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ORIGINAL RESEARCH PAPER

Pathological Aspects of Trombidiosis in Crested Lark (*Galerida Cristata*)

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Abstract

Trombidiosis represents an ectoparasitic infestation by acarian larvae which belongs to *Trombiculidae* family that could affect different animal hosts around the world. In April 2016 in the farms around Zabol district in Southeast of Iran, 12 adult Crested Larks (*Galerida cristata*) were found with multiple bright orange nodular lesions on the skin of the neck, wings, thoracic area and the limbs. Crested lark (*Galerida cristata*) is the bird that belongs to *Alaudidae* family and is well represented in Europe, Middle East and Asia. The bird is usually seen on the ground near roadsides in many areas of Iran. The Trombiculid mite larva was detected in the lesions. Histopathological evaluation showed orthokeratotic hyperkeratosis, epidermal hyperplasia, hydropic degeneration and epidermal necrosis. Also, inflammatory cells penetration into the dermis was notable. The present study is the first illustration of pathological findings in the skin lesions of affected Crested Larks with *Trombicula* harvest mite.

1. Introduction

Crested lark (*Galerida cristata*) is the bird belongs to *Alaudidae* family that is well represented in Europe, Middle East and Asia. The adults are generally quiet and small, 17-19 cm in length and 35-50 gram in weight. The bird is usually seen on the ground near roadsides in many areas of Iran (Khaleghizadeh et al., 2005). Trombidiosis represents an ectoparasitic infestation by acarian larvae which belongs to *Trombiculidae* family. The only larval stage of 50 known species of this parasite could attack domestic animals and humans around the world (Wall and Shearer, 2001; Polina, 2012). Present study illustrates pathological aspects of infestation with *Trombiculidae* harvest mites in the Crested lark in Zabol district, Sistan and Balouches-

tan, Iran.

2. Materials and Methods

In April 2016, the present case study was conducted on 30 numbers of adult Crested Larks in the farms around Zabol region in the north of Sistan and Balouchestan province of Iran (Fig. 1). This province is located at approximately 487 m above sea level. It has a desert climate (Köppen-Geiger classification: BWh), with an annual rainfall of <7 cm and long and windy summers in which temperatures frequently exceed 42°C. Twelve numbers of investigated birds were found with multiple bright orange nodular bumps approximately 2-3 mm in diameter on the skin of the neck, wings, thoracic area and posterior part of the limbs (Fig. 1).

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The mites were collected directly and preserved in vials with ethanol 70% for identification and morphological studies with light microscope. The taxonomic key (Brennan and Goff, 1977) was used for identification and classification of the mites. For histopathological examination, skin biopsies from five of the birds were collected and fixed in 10% buffered formalin. Then, the samples were embedded in paraffin, sectioned at five μ m and were stained by hematoxylin and eosin method.



Fig. 1. *Galerida cristata* with multiple bright orange nodular lesions on the leg.

3. Results

Infected birds did not show any clinical symptoms. There was also no difference in the severity of infections and the number of lesions in the infected organs. Histopathological evaluation revealed that reaction to mites was confined to the epidermis and superficial dermis associated with orthokeratotic hyperkeratosis, epidermal hyperplasia (especially acanthosis), hydropic degeneration and epidermal necrosis (Fig. 2) and (Fig. 3).

4. Discussion

A few studies investigated integumentary lesions caused by chigger mites. Cunningham et al. (2001) described trombiculiasis cutaneous lesions in epidermis and superficial dermis of the Florida black bear that is similar to our research on Crested Lark. Although, in comparison to their report, ulceration with heterophile aggregation were not observable in the present study. Moreover, our investigation showed that the dermis was diffusely infiltrated with mononuclear inflammatory cells predominantly macrophages, lymphocytes and occasionally plasma cells and fibroblasts. These

findings are in accordance with study by Mendez et al. (2010). In another study, mix ectoparasitic infestation of the side-blotched lizard was evaluated histopathologically and in chigger mites infested cases, epidermal necrosis associated with epithelial cells hyperplasia were prominent lesions (Goldberg and Bursey, 1991). Like the present report, inflammatory cells infiltration into the dermis was noticeable in the study. Thus, the observed lesions are in accordance with our findings. However, heterophils were not found in our research. Other microscopic findings obtained from the present study are in accordance with which were described in some literatures (Little et al, 1997; Brown et al, 2006; Spieler and Linsenmair, 2016). Several researches have reported trombidiosis in various hosts (Stekolnikov et al, 2007; Gomez-Puerta et al, 2012) but there are no reports found in literature about pathological aspects of trombidiosis in Crested Lark.

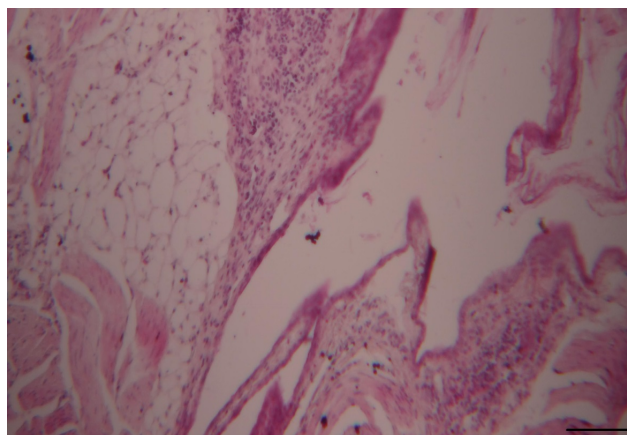


Fig. 2. Skin, *Galerida cristata*. Orthokeratotic hyperkeratosis and infiltration of mononuclear inflammatory cells into the epidermis (H&E, Bar= 100 μ m).

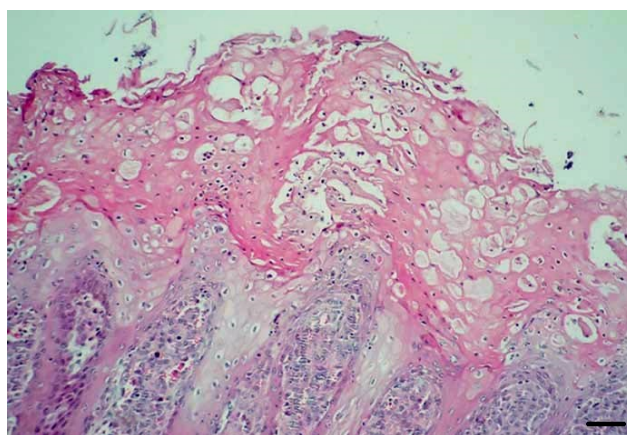


Fig. 3. Skin, *Galerida cristata*. Epidermal hyperplasia (acanthosis) and necrosis associated with hydropic degeneration (H&E, Bar= 50 μ m).

5. Conclusion

The results of this study illustrated that although, histopathological lesions of chigger mite were not at acute phase in the bird and heterophilic infiltration was not prominent but immune-inflammatory responses to the mite were significant.

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The authors declare that there is no conflict of interest.

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ORIGINAL RESEARCH PAPER

The Protective Effect of Thiamine on the Histo-stereological Structural Changes of the Testicles and Oxidative State Induced by Copper Oxide Nanoparticles in Rat

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Abstract

Nanoparticles represent an innovative and highly promising technology in the fields of medicine and diagnostics, offering unique capabilities for targeted drug delivery and advanced diagnosis. This study aimed to investigate the protective effects of thiamine against the histo-stereological and biochemical alterations induced by copper oxide nanoparticles (CuO NPs) in the testes. A total of 72 adult male, healthy Wistar rats were divided into four groups: control, thiamine (30 mg/kg), CuO NP (25 mg/kg), and a combination of CuO NPs and thiamine (30 mg/kg + 25 mg/kg). All substances were administered via intraperitoneal injection, once daily. After 14 days of treatment, the tissue and blood samples were collected for histo-stereological evaluation and total antioxidant capacity (TAC), malondialdehyde (MDA), superoxide dismutase (SOD) catalase (CAT) antioxidant enzymes, body weight and testicular weight assessment. Control and thiamine groups maintained normal seminiferous tubule structure, regular spermatogenesis, and high antioxidant levels. CuO NPs administration induced severe histopathological damages, including significant reduction in seminiferous tubule diameter, disruption of the germinal epithelium, a significant decrease in the number of spermatocytes and spermatids, a significant decrease in body and testicular weight, and diminished antioxidant enzyme activity ($P < 0.05$). Thiamine exerts protective effects in testicular tissue through its antioxidant, anti-inflammatory and anti-apoptotic properties, and may serve as an effective adjunct therapeutic agent to counteract the reproductive toxicity and adverse effects induced by CuO NPs.

1. Introduction

CuO NPs constitute a significant class of inorganic nanomaterials distinguished by a suite of unique physicochemical attributes including specific optical, thermal, and magnetic properties, in addition to enhanced electrical conductivity, high catalytic activity, and a substantial surface-to-volume ratio. Notably,

CuO NPs demonstrate a potent bactericidal effect coupled with considerable antiviral and antifungal efficacy, underscoring their utility in biomedical and therapeutic domains. Current research is extensively exploring their role in wound healing protocols, advanced drug delivery systems, and as cytotoxic agents in oncology treatments (1–6). Despite their increasing utility, the pervasive application of CuO NPs necessitates a crit-

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ical evaluation of potential toxicological profile, particularly concerning reproductive health. Exposure to CuO NPs has been shown to induce detrimental effects on both the male and female reproductive systems. The mechanism of action frequently involves the induction of oxidative stress, inflammatory responses, generalized cellular damage, and apoptosis. Specifically, the generation of reactive oxygen species (ROSs), often exceeding the endogenous antioxidant capacity of the organism, precipitates substantial peroxidation of lipids and degradation of proteins. This resultant oxidative damage critically impairs spermatogenesis, leading to a measurable reduction in sperm count, diminished motility, and compromised overall semen quality. Furthermore, elevated ROSs levels can induce DNA fragmentation, which fundamentally jeopardizes male fertility. In the testes, inflammation mediated by CuO NPs can severely restrict spermatogenesis, induce structural damage to the seminiferous tubules, and alter the integrity of the blood-testis barrier function, culminating in infertility (3, 7, 8).

Beyond cellular damage, CuO NPs possess the capacity to interfere with the endocrine system by disrupting androgenic hormone regulation. Such disruption can manifest as reduced libido, decreased spermatogenic output, and potentially contribute to erectile dysfunction. The induction of apoptosis within spermatogenic cells further exacerbates the decline in germ cell populations (3, 7, 8). For maternal exposures, the potential for placental barrier penetration by CuO NPs raises significant concerns, as this can lead to fetal growth restriction, congenital anomalies, and developmental deficits in the offspring, with particular vulnerability noted in the development of the reproductive organs (3, 7, 8).

Thiamine (Vitamin B1) is an essential water-soluble vitamin, crucial for the development and physiological function of various cell types. Its metabolic significance is profound, serving as a coenzyme in key pathways, including energy metabolism, lipid synthesis, and nucleotide formation. It is also critically involved in neural cell development and enhances endothelial cell function (9). Crucially, thiamine exhibits inherent antioxidant properties. It acts as an essential cofactor under physiological stress, providing demonstrated protection against lead-induced lipid peroxidation in hepatic and renal tissues. Functioning as an effective scavenger of superoxide and hydroxyl anion free radicals, thiamine significantly augments the cellular response mechanisms to oxidative stress. Preclinical models have shown that thiamine effectively mitigates nanoparticle-induced nephrotoxicity (9).

Acknowledging that CuO NPs induce reproductive toxicity via oxidative stress and inflammatory cascades, and given the established antioxidant properties of thiamine, this investigation will meticulously examine the resulting histo-stereological and oxidative dam-

age in the testicles, while simultaneously assessing the potential ameliorative effects of thiamine.

2. Materials and Methods

CuO NPs, supplied by Sigma-Aldrich (USA), were used. The manufacturer specified a 99% purity, particle size of approximately 50 nm, a density of 6.4 g/cm³, a specific surface area of 20 m²/g, and appear as black spherical crystalline structures.

3. Animal Experimental Design and Protocols

A total of 72 healthy adult male Wistar rats, weighing between 200–250 grams, were utilized in accordance with established institutional and international animal care and use guidelines. They were maintained in standard polycarbonate cages under controlled environmental conditions, specifically a 12-hour light/12-hour dark cycle, a stable ambient temperature of 22±2°C, and a relative humidity of 55%–60% (23).

Prior to the commencement of the experiments, animals underwent a one-week period of acclimatization. Following this period, they were randomly allocated into four experimental groups, with each group comprising 18 animals (three replicates with n=6 each). All substances were prepared using sterile normal saline, and administered via intraperitoneal injection for a duration of two consecutive weeks, with injections performed once daily. The volume of administration for all injections was consistently 1 mL (23).

Group 1 served as the control and received only normal saline, whereas Groups 2, 3, and 4 were treated with 30 mg/kg of thiamine, 25 mg/kg of CuO NPs, and a combination of 30 mg/kg of thiamine and 25 mg/kg of CuO NPs, respectively. The doses of 25 mg/kg for CuO NPs and 30 mg/kg for thiamine were chosen based on previous experimental studies. CuO NPs at 25–35 mg/kg have been shown to induce testicular oxidative damage in rodents (Naz et al., 2023). Thiamine at comparable intraperitoneal doses has demonstrated antioxidant and protective effects in rodent models of oxidative stress (Amiri et al., 2018). The suspension of CuO NPs was vigorously sonicated for 30 minutes immediately prior to each daily administration to ensure a homogeneous dispersion. The pH of all final solutions and suspensions was carefully adjusted to 7.0–7.4.

At the end of the 14-day treatment period, rats were weighed using a digital analytical balance (accurate to 0.01 g) prior to euthanasia. Both testes were carefully excised from the scrotum, gently cleaned of surrounding tissues, and weighed individually using the same balance. All weights were recorded as fresh, unfixed tissue immediately after dissection to ensure accuracy. Upon completion of the 14-day experimental period,

blood samples for subsequent biochemical analysis were collected from all animals via cardiac puncture (24). Following blood collection, the rats were immediately euthanized through the intraperitoneal administration of 150 mg/kg of sodium pentobarbital (Sigma-Aldrich, USA), and the testes were then carefully excised from the scrotal sac for further tissue analyses (23).

4. Stereology

After excision of the testes and careful removal of surrounding tissues, the primary volume (V_{primary}) of each testis was determined using the immersion method (25). To achieve isotropic uniform random (IUR) sampling, the Orientator method was applied. Ten serial sections with a thickness of 5 μm were randomly selected per testis (25, 9).

For stereological analysis, ten slides per animal were examined, and within each slide, ten non-overlapping fields of view (FOV) were captured at $400\times$ magnification, resulting in a total of 100 FOV per animal (25, 9). Volume densities (V_v) of seminiferous tubules, interstitial tissue, primary spermatocytes, and spermatids were quantified using the point-counting method, calculated as the ratio of points hitting the structure of interest to the total number of test points (25).

Tissue processing was performed using an automated processor (Leica TP 1020, Germany), and sections were stained with Hematoxylin and Eosin (H&E) for standard histological evaluation. Ten randomly selected slides per animal were examined, and within each slide, ten distinct fields of view were captured, resulting in 100 FOV per animal. Quantitative measurements included seminiferous tubule diameter, germinal epithelium height, and volume densities of seminiferous tubules, interstitial tissue, primary spermatocytes, and spermatids. All measurements were independently performed by two blinded observers, and any discrepancies were resolved through joint reevaluation to ensure accuracy and reproducibility (25, 9).

After excision of the testes and careful removal of surrounding tissues, the primary volume (V_{primary}) of each testis was determined using the immersion method (25). To achieve isotropic uniform random (IUR) sampling, the Orientator method was applied. Ten serial sections with a thickness of 5 μm were randomly selected per testis (25,9).

5. The Number of Cells and the Diameter of the Tubes

For the quantification of spermatogenic cells and measurement of seminiferous tubule diameter, the same sections used for stereological analysis were examined. Ten randomly selected slides per animal were analyzed, and within each slide, ten non-overlapping fields of

view (FOV) were systematically examined, resulting in 100 FOV per animal (25, 9).

Primary spermatocytes and spermatids were identified and counted based on established morphological criteria and their relative positions within the seminiferous tubules (9). Seminiferous tubule diameter was measured using a calibrated ocular micrometer at $400\times$ magnification, consistent with the magnification used for stereological assessment (25, 9).

All stereological parameters, including volume densities of seminiferous tubules, interstitial tissue, primary spermatocytes, and spermatids, were recorded for each tubule. This approach ensured that measurements of tubule diameter and cell counts were directly correlated with stereological observations.

All measurements and cell counts were independently performed by two blinded observers to prevent bias. Any discrepancies were resolved through joint reevaluation to ensure accuracy and reproducibility (25, 9). The resulting mean values provided reliable and reproducible data for the assessment of testicular structure, spermatogenic integrity, and the potential protective effects of thiamine against CuO NP-induced damage.

6. Antioxidant Enzyme Assessment

A colorimetric technique utilizing commercial kits (Navand Salamat, Urmia, Iran) was employed to quantify Catalase activity. The reaction mixture consisted of 30 mM phosphate buffer (pH 7.0), 2.9 mL of hydrogen peroxide, and 0.1 mL of serum. The consumption of hydrogen peroxide was monitored, and the absorbance was measured at a wavelength of 550 nm (UNICO, SQ 2800, China). Catalase activity was expressed in units per liter (U/L).

SOD Enzyme activity was measured using commercial kits (Navand Salamat, Urmia, Iran), based on the inhibition of pyrogallol autoxidation and expressed as units per liter (U/L).

MDA is derived from the decomposition of polyunsaturated fatty acids. A colorimetric assay was employed, based on the interaction between MDA and thiobarbituric acid (TBA) to form a pink-colored complex that can be measured spectrophotometrically at 532 nm. The TBA reagent was prepared by mixing equal volumes of a 15% (v/v) trichloroacetic acid (TCA) solution, a 0.375% (v/v) thiobarbituric acid solution, and 0.25 N hydrochloric acid. A 2 mL aliquot of the reagent was added to 1 mL of serum, and the mixture was incubated in a boiling water bath for 50 minutes. After incubation, the sample was cooled for 10 minutes, then centrifuged at 1000 g for 10 minutes at 4°C . The absorbance of the supernatant was measured at 532 nm (UNICO, SQ 2800, China). The concentration of MDA was determined using the molar

absorption coefficient in phosphate buffer (6).

The ferric reducing antioxidant power (FRAP) method was used to quantify Total Antioxidant Capacity using spectrophotometry at a wavelength of 593 nm (UNICO, SQ 2800, China). This method was based on the reduction of ferric ions to ferrous ions by the reducing power of antioxidant compounds present in plasma. The results are expressed as micromoles per liter (mol/L).

7. Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). Prior to statistical comparisons, datasets were tested for normality using the Shapiro–Wilk test and the homogeneity of variances among groups was assessed using Levene’s test. Once the assumptions of normality and equal variances were confirmed, one-way analysis of variance (ANOVA) was performed, followed by Tukey’s post-hoc test for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

8. Results

8.1. Histomorphometry

Histological examination of the control group revealed a typical, compact architecture within the testicular tissue. The seminiferous tubules were maintained a dense structure and exhibited a demonstrably normal spermatogenesis process. Specifically, the reproductive epithelium lining the seminiferous tubules displayed a regular, stratified arrangement. Proper cell-to-cell junctions were clearly discernible between the different germinal cell populations. The cellular layers were organized in the expected sequential order, consisting of basal spermatogonia, followed by spermatocytes, spermatids, and finally, spermatozoa (mature sperm cells). The intact cellular connectivity and the sequential stratification of the germ cells within these tubules serve as evidence of the uninterrupted differentiation of the primary germ line into mature gametes (Figs. 1(A) and 2(A1)).

Histological analysis of the group treated with thiamine demonstrated testicular morphology highly comparable to that of the control group. The seminiferous tubules maintained normal architecture and the germinal cells within were arranged in a regular, orderly fashion. The unimpaired spermatogenic process was observed, exhibiting a clear and sequential organization of the germ cell line. The tubules were well-defined, containing a substantial population of germ cells, which included the full sequence of development from spermatogonia through to spermatozoa. Additionally, the interstitial tissue presented a normal configuration, and the intercellular spacing within the

tubules appeared normal. Overall, the thiamine group showed no significant deviation from the control, confirming preserved spermatogenesis and structural integrity (Figs. 1(B) and 2(A2)).

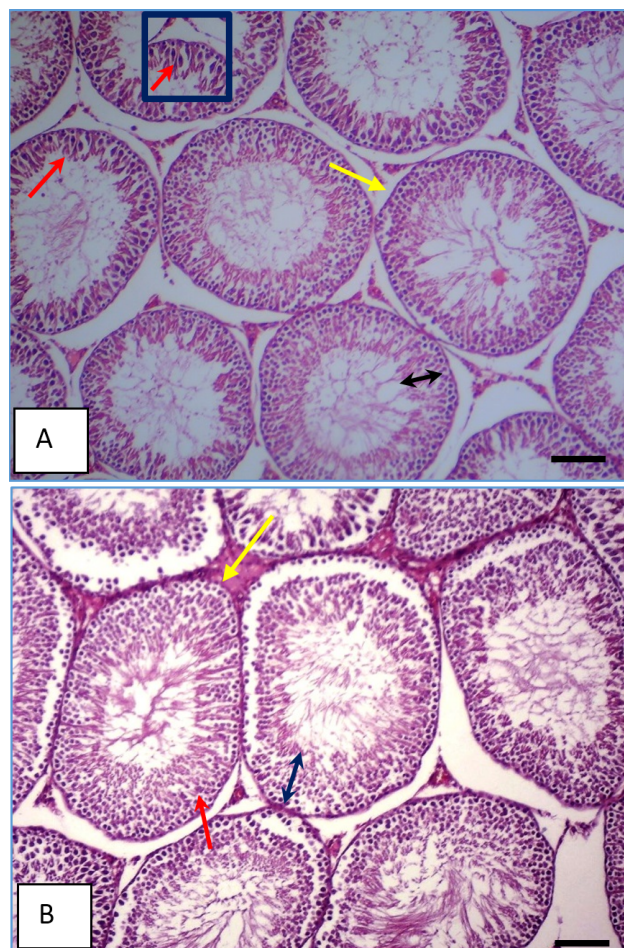


Fig. 1. Light microscope photomicrograph of the testicular tissue from adult mice in the control A) and thiamine B) groups; a normal shape of the seminiferous tubules (yellow arrow) with a normal germ wall (black arrow) along with the germ cell line “primary spermatocytes” (red arrow with magnification) is observed; (magnification 200 $\times$, H&E staining).

CuO NPs-treated group revealed severe structural and cellular anomalies within the testicular tissue, indicating significant spermatogenic disruption. The seminiferous tubules exhibited marked irregularities and disorganization. A notable finding was the premature sloughing of spermatogonia, spermatocytes, and spermatids into the tubular lumen, frequently accompanied by a marked reduction in the density of these germ cell populations. Crucially, the basal integrity of the tubules was compromised, evidenced by the separation and degeneration of spermatogonia from the basement membrane. These pathological changes resulted in the irregularity and disorganization of the reproductive epithelium, leading to a noticeable decrease in epithelial

height and a corresponding enlargement of the seminiferous tubule lumen. Furthermore, the interstitial tissue surrounding the tubules was distinctly edematous. Signs of acute inflammation were also evident, including vascular congestion and widespread cellular damage, characterized by eosinophilic cytoplasm and pyknotic nuclei (Figs. 2(B1) and B2).

In the CuO NPs + Thiamine group, the majority of the destructive changes and irregularities induced by CuO NPs were reversed and ameliorated. As a result, the reproductive epithelium of the seminiferous tubules exhibited an almost normal structure (Figs. 2(C1) and C2).

8.2. Stereology

The diameter of the seminiferous tubules in the CuO NPs group was significantly reduced compared to the

control group. Conversely, the CuO NPs + Thiamine group showed a significant increase in tubule diameter when compared specifically to the CuO NPs group ($P < 0.05$). Regarding stereological parameters (Table 1), the CuO NPs group exhibited, a significant decrease in the mean volume density (V_v) of the seminiferous tubules ($60.25\% \pm 5.36$) compared to the control group ($86.45\% \pm 6.39$) ($P < 0.05$). Correspondingly, the V_v of the interstitial tissue was significantly increased in the CuO NPs group ($36.17\% \pm 1.67$) relative to the control ($9.43\% \pm 3.27$) ($P < 0.05$), reflecting severe tissue damage and edema. Furthermore, the V_v of the germinal cells, primary spermatocytes ($17.34\% \pm 2.27$) and spermatids ($21.2\% \pm 1.15$), were both significantly decreased in the CuO NPs group compared to the control ($32.4\% \pm 3.01$ and $45.6\% \pm 4.25$) respectively ($P < 0.05$).

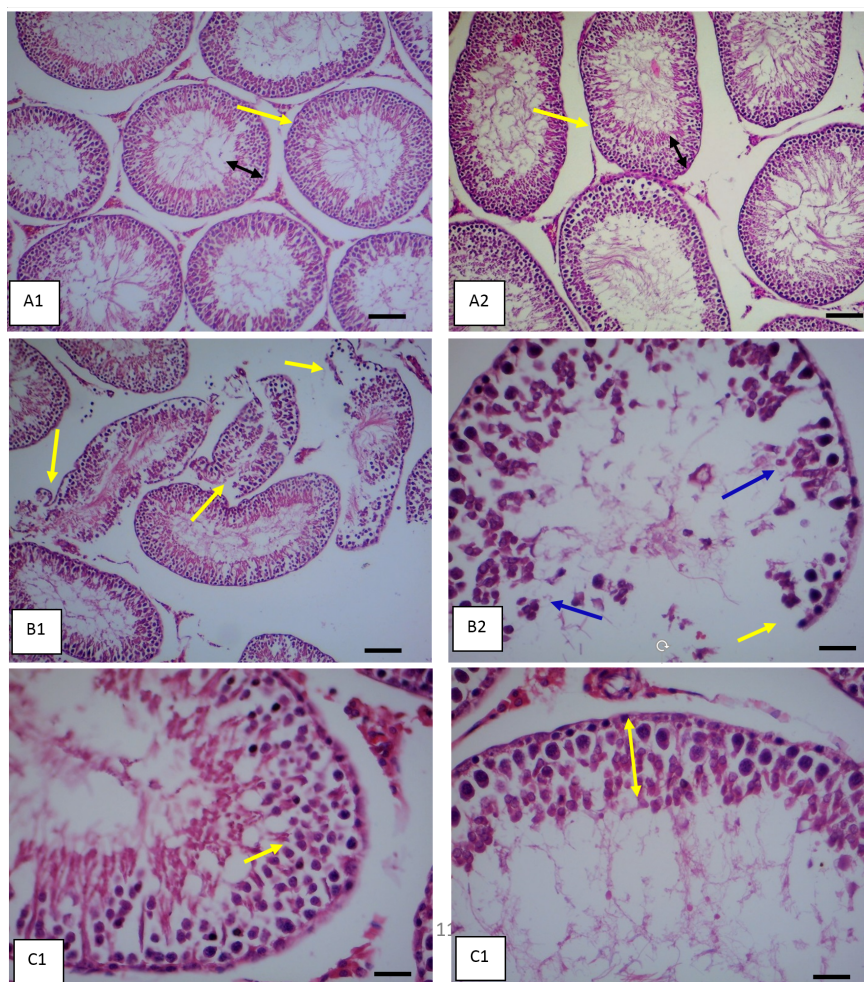


Fig. 2. Light microscope photomicrograph of testicular tissue from adult mice in the control (A1, A2), CuO NPs (B1, B2) and CuONPs+ Thiamine (C1, C2) groups. In the control group (A1, A2), the complete histological structure of the seminiferous tubules (yellow arrow) with a normal germinal wall (black arrow) has been seen. In the CuO NPS group (B1, B2), the germinal epithelium of the seminiferous tubules lost its integrity (yellow arrow) and the spermatogenic cells of the germinal wall had disintegrated (blue arrow). In the CuO NPs+ Thiamine (C1, C2) group, the germinal epithelium had largely maintained its normal structure and was improved (yellow arrow; magnification 200 $\times$, H&E staining).

Table 1

Mean volume densities (%) of seminiferous tubules, interstitial tissue, and germinal cells in all experimental groups.

Group	Group			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
Parameters				
Volume density (%)				
Seminiferous tubules	86.45±6.39 ^a	82.48±6.34 ^a	60.25±5.36 ^b	79.32 ± 6.54 ^a
Interstitial tissue	9.43±3.27 ^a	10.36±2.89 ^a	36.17±1.67 ^b	9.87±2.71 ^a
Primary spermatocyte	32.4±3.01 ^a	28.14±3.11 ^a	17.34±2.27 ^b	22.5±1.19 ^a
Spermatids	45.6±4.25 ^a	40.2±3.96 ^a	21.2±1.15 ^b	31.3±2.75 ^a

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

Table 2

Mean number of spermatocytes, spermatids, and seminiferous tubule diameter (m) in all experimental groups.

	Group			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
Diameter (μm)				
Tubules	242±26 ^a	240±25 ^a	179±20 ^b	237±18 ^a
Number				
Spermatocyte	23.4±2.04 ^a	22.1±1.36 ^a	11.6±1.02 ^b	18.7±2.15 ^a
Spermatid	47.2±3.58 ^a	53.9±4.68	17.3±3.81 ^b	38.4±4.60 ^a

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

Table 3

Mean body weight and testes (gr) and diameter seminiferous tubules (μm) in all groups

	Group			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
Parameters				
Weight (gr)				
Testis	1.78±0.08 ^a	1.71±0.08 ^a	1.28±0.07 ^b	1.62±0.09 ^a
Body	210.4±4.3 ^a	224.4±4.4 ^a	171.3±3.85 ^b	207±4.5 ^a

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

In contrast, treatment with thiamine significantly mitigated these adverse effects. The CuO NPs + Thiamine group demonstrated a significant increase and structural improvement in the Vv of the seminiferous tubules (79.32%±6.54), primary spermatocytes (22.5%±1.19), and spermatids (31.3%±2.75) compared to the CuO NPs group (P< 0.05). This recovery was concurrently reflected by the significant decrease in the Vv of the interstitial tissue (9.87% ±2.71) in the combined treatment group compared to the CuO NPs group (P<0.05) (Table 2).

Different letters (a&b) indicate significant differences between different treatment groups (P< 0.05); data are presented as Mean ± SEM.

Additionally, there was a significant decrease in the number of spermatocytes and spermatids in the CuO NPs group compared to the control (P<0.05). In contrast, a significant increase in the number of spermatocytes and spermatids was noted in the CuO NPs + Thiamine group compared to the CuO NPs group

(P<0.05) (Table 2).

8.3. Body and Testicle Weights

At the conclusion of the experiments, analysis revealed no significant difference in the mean body weight or mean testicular weight among the Control, Thiamine-alone, and the CuO NPs+Thiamine co-administration groups (p>0.05). Conversely, the CuO NPs group exhibited a significant decrease in both mean body weight and mean testicular weight when compared to all other experimental groups (p<0.05).

8.4. Antioxidants Assesement

The Control and Thiamine-alone groups did not differ statistically in the measured CAT enzyme activity (0.05). Conversely, both the CuO NPs and the CuO NPs + Thiamine groups exhibited a significant decrease in enzyme activity when compared to both the Control and Thiamine groups (0.05) (Table 4).

Table 4

CAT changes (nm/ml) in the experimental groups.

	Groups days			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
14	176.7±11.89 ^a	178.2±12.18 ^a	145.2±8.36 ^b	150.4±10.01 ^b
<i>P value</i>	<i>p>0.05</i>	<i>p>0.05</i>	<i>P<0.05</i>	<i>p>0.05</i>

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

Table 5

SOD changes (nm/ml) in the experimental groups.

	Groups days			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
14	22/74±3/57 ^a	22/74±3/57 ^a	41/52±5/37 ^b	29/71±4/28 ^b
<i>P value</i>	<i>p>0.05</i>	<i>p>0.05</i>	<i>P<0.05</i>	<i>p>0.05</i>

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

Table 6

TAC changes (nm/ml) in the experimental groups.

	Groups days			
	Control	Thiamine	CuO NPs	CuO NPs+ Thiamine
14	0.12±0.02 ^a	0.10±0.02 ^a	0.08±0.01 ^b	0.11±0.02 ^a
<i>P value</i>	<i>p>0.05</i>	<i>p>0.05</i>	<i>P<0.05</i>	<i>p>0.05</i>

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

Table 7

MDA changes (nm/ml) in the experimental groups.

	Groups days			
	Control	Thiamine	CuONPs	CuONPs+ Thiamine
14	1.39±0.65 ^a	1.42±0.76	3.67±0.37 ^b	1.87±0.49 ^a
<i>P value</i>	<i>p>0.05</i>	<i>p>0.05</i>	<i>P<0.05</i>	<i>p>0.05</i>

a/b letters: Different letters indicate statistically significant differences between groups (P<0.05)

The SOD activity was not significantly differ neither between control and thiamine nor between CuO NPs and CuO NPs + Thiamine groups ($p>0.05$). However, it was significantly higher in the CuO NPs and CuO NPs + Thiamine groups than control and thiamine groups ($p<0.05$) (Table 5).

No significant difference was seen between control, thiamine and CuO NPs + Thiamine in the TAC ($P>0.05$). However, it was significantly lower in the CuO NPs group than other experimental groups ($P<0.05$) (Table 6).

No significant difference was seen between control, thiamine and CuO NPs + Thiamine in the MDA level ($P>0.05$). Administration of CuO NPs resulted in a significant increase in the level of MDA compared to the control, thiamine and CuO NPs + Thiamine groups ($P<0.05$). In contrast, the (CuO NPs + Thiamine) group exhibited a significantly lower MDA level compared to the CuO NPs group ($P<0.05$) (Table 7).

9. Discussion

In this study, exposure CuO NPs resulted in a significant decline in body weight and testicular mass.

Nanoparticles such as CuO NPs, owing to their extremely small size, can readily traverse cellular membranes including barriers such as the blood-testis barrier and accumulate in tissues, triggering oxidative stress and inflammatory responses that can lead to hepatotoxicity, neurotoxicity, and reproductive injury. Gastrointestinal absorption and accumulation of CuO NPs may also provoke digestive disturbances, impaired nutrient uptake, anorexia, negative energy balance, and consequential weight loss (1, 2).

In addition to the direct oxidative and inflammatory effects of CuO NPs on testicular tissue, the observed reduction in body weight likely contributed to the decreased testicular weight and spermatogenic damage. Systemic weight loss may reflect impaired nutrient absorption, lower energy availability, and reduced blood flow to peripheral organs, including the testes (1,3,4). These factors can exacerbate testicular atrophy and make germ cells more susceptible to oxidative stress, further compromising spermatogenesis. Therefore, the combined impact of systemic energy deficiency and direct nanoparticle-induced oxidative damage provides a more comprehensive explanation for the reductions in testis mass and germ cell

populations observed in the CuO NPs group.

The reproductive system is especially vulnerable to nanoparticulate toxicity. Germ cells undergo rapid mitotic and meiotic divisions and have elevated metabolic demands, which amplify their sensitivity to toxic insults. Moreover, the nanoscale transformation of CuO particles alters their physicochemical properties—such as increased surface area, solubility, and membrane permeability thereby facilitating intracellular uptake and intensifying cytotoxicity (3).

Accumulation of nanoparticles within testicular tissue can induce histological damage; apoptosis, necrosis, and degenerative changes, that disrupt the spermatogenic lineage and reduce populations of spermatogonia, spermatocytes, and spermatids. Because testis mass is largely a function of differentiated germ cells and seminiferous epithelium, suppression of spermatogenesis, impairment of Sertoli cell support, or reduction in seminiferous tubular fluid can all contribute to testicular weight loss. In addition, interference with androgen production or anti-androgenic effects of CuO NPs may further reduce the weight of accessory reproductive organs (4, 5).

CuO NPs may disrupt endocrine regulation by inhibiting pituitary gonadotropins (FSH and LH), thereby hindering testosterone synthesis, a hormone critical for spermatogenesis and maintenance of accessory sex organs. Elevated ROSs and protein oxidation can damage Leydig cells, while downregulation of steroidogenic acute regulatory protein (StAR) expression can limit cholesterol transport into mitochondria, further impairing steroidogenesis (6).

Oxidative stress is a predominant mechanism underlying CuO NP toxicity. These nanoparticles provoke a concentration-dependent increase in intracellular ROSs, which can oxidatively damage DNA, proteins, and lipids. Dissolution of CuO NPs releases free copper ions that participate in redox cycling, further amplifying ROSs production. This oxidative onslaught depletes cellular antioxidants e.g. glutathione, catalase, superoxide dismutase, elevates lipid peroxidation such as increased MDA, and ultimately precipitates cell injury and apoptosis (7, 8). Additionally, oxidative modifications such as protein carbonylation may disrupt normal cellular processes, and sustained activation of inflammatory pathways can exacerbate tissue damage (9).

Our results indicate that thiamine can ameliorate many of these deleterious effects. As an antioxidant, thiamine may enhance the activity of key enzymes e.g. catalase and SOD, scavenge free radicals, reduce lipid peroxidation, thus lowering MDA, and attenuate inflammatory responses, thereby helping to maintain cellular integrity in reproductive tissues (10, 11). Thiamine's role as a ligand in transport processes may facilitate its cellular uptake and antioxidant defense against ROSs and nanoparticle-induced stress (10).

Beyond its redox-modulating effects, thiamine might contribute to restoring hormonal balance disrupted by nanoparticle exposure. By decreasing oxidative stress, it may stabilize gene expression within steroidogenic pathways and support hormone synthesis, thus mitigating disturbances in the hypothalamic-pituitary-gonadal axis. Thiamine's antioxidant properties may also protect signaling pathways in Leydig, Sertoli, and germ cells from ROSs-induced dysregulation, preserving functions essential for spermatogenesis and endocrine homeostasis (12).

10. Conclusion

Exposure to CuO nanoparticles provoked significant histological and stereological damage in male reproductive tissues, including reduced seminiferous tubule diameter, lowered sperm density, disorganization of the reproductive epithelium, enlarged tubular lumens, interstitial edema, and depletion of spermatogenic cell populations, primarily attributable to oxidative stress and ROSs-mediated injury. Thiamine administration alleviated CuO NP-induced reproductive damage by preserving testicular structure and function through its antioxidant, anti-inflammatory, and endocrine-stabilizing effects, suggesting its potential as a therapeutic agent against nanoparticle-induced toxicity.

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Conflict of Interest Disclosure

The authors declare that they have no conflicts of interest.

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REVIEW RESEARCH PAPER

Hamburger Standard in Iran: Mini Review

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Abstract

One of the most important types of meat product is the hamburger, which is particularly popular due to the use of different percentages of meat and vegetable proteins. Hamburg, Germany, is considered the birthplace of the hamburger. In the 19th century, the plan to make hamburgers was formed in this city. For the first time, they ground beef and mixed it with vegetables, onions, salt and pepper and served it in molds without bread, thus obtaining the Hamburger Steak. The standards for the production and testing of hamburger in Iran include standards 2304 (Raw frozen hamburger - Specifications and test methods) and 6103 (Detection of unauthorized animal tissue by histological method). These standards address the formulation of hamburger, their classification, chemical properties, microbial properties, histological properties, and packaging. The National Standards Organization of Iran is the one that formulates the laws and regulations related to standards and implements and supervises them. The Standards Organization of Iran has a governmental structure as a subsidiary of the presidential institution. Almost all of the country's gross domestic products, especially in the field of food, have the standard mark engraved next to the Food and Drug Administration's health apple.

1. Introduction

One of the most important types of burgers is the hamburger, which is particularly popular due to the use of different percentages of meat and vegetable proteins. The hamburger production process includes preparing and weighing raw materials, mixing and molding. First, the raw materials must be of appropriate quality. In fact, quality control of raw materials is of great importance. Meat preparation includes: dividing the carcass, removing inedible entrails, cleaning the meat, deboning the meat, and finally grinding the meat. After preparation, the hamburger is stored in sub-zero cold stores. Hamburg, Germany, is considered the birthplace of the hamburger. In the 19th century, the plan to make hamburgers was formed in this city. For the first time, they ground beef and mixed it with veg-

etables, onions, salt and pepper and served it in molds without bread, thus obtaining the Hamburger Steak (National Standard of Iran No. 2304-2016). Hamburger is subject to mandatory standard regulations in the National Standards Organization of Iran (Asadi *et al.*, 2017).

2. Definition of Hamburger Standard

According to National Standard 2304, frozen raw hamburger is minced meat from halal animals to which other permitted ingredients have been added and prepared and produced by special mechanical devices in the production unit and packaged and labeled in a tablet form under hygienic conditions and supplied frozen. In the hamburger production standard in Iran, we have a type of burger mixed with meat and veg-

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etable proteins that is permitted and in which the minimum meat content is 30% using vegetable proteins and other permitted ingredients. Permitted vegetable proteins include soybean and gluten (Asadi *et al.*, 2023).

3. Types of Hamburgers According to Iranian Standards

3.1. *Hamburger with 30% Red Meat*

Hamburger mixed with meat and vegetable proteins (soybean and gluten).

3.2. *Hamburger with 60% Red Meat*

A type of hamburger in which a minimum of 60% meat is allowed along with other ingredients, and the use of vegetable proteins is prohibited.

3.3. *Hamburger with 75% Red Meat*

A type of hamburger in which a minimum of 75% meat is allowed along with other ingredients, and the use of vegetable proteins is prohibited.

3.4. *Hamburger with 85% Red Meat*

A type of hamburger in which a minimum of 85% meat is allowed along with other ingredients, and the use of vegetable proteins is prohibited.

3.5. *Hamburger with 90% Red Meat*

A type of hamburger in which a minimum of 90% meat is allowed along with other ingredients, and the use of vegetable proteins is prohibited.

3.6. *Hamburger with 95% Red Meat*

A type of hamburger in which the minimum meat content is 95% along with other ingredients is allowed and the use of vegetable proteins is prohibited (Yarvari *et al.*, 2025).

4. Main Requirements of the Hamburger Standard

4.1. *Raw Materials*

The raw materials include the following:

4.1.1. Red Meat

Must be made from beef or mutton that is prepared under veterinary supervision and under hygienic conditions and healthy slaughter. The use of chicken dough is prohibited.

4.1.2. Edible Oil

4.1.3. Fillers and Binders

4.1.4. Permitted Edible Phosphates

4.1.5. Edible Salt

4.1.6. Vegetable Proteins

Only includes vegetable proteins of soybean and gluten.

4.1.7. Vegetables and Spices (Ghazanfari *et al.*, 2018)

5. Sensory and Physical Characteristics

Sensory and physical characteristics include color, odor and texture, and the final product must have its natural color, odor and texture (Iranian National Standardization Organization 2304, 2016).

6. Chemical Properties

In the Iranian standard, the items tested for the hamburger product include measuring protein, fat, moisture, ash, starch and phosphate.

7. Microbiological Properties

The microbial items tested in this product include total microbial count, *Escherichia coli*, mold and yeast, *Salmonella*, *Staphylococcus aureus*.

8. Prohibitions and Prohibited Items in Hamburgers

The use of artificial colors in hamburgers is prohibited, such as red color, which is used to make the hamburger look good. The use of preservatives exceeding the standard limits in meat products is prohibited, sulfites and boric acid and nitrates and nitrites in high amounts cause allergic reactions and ultimately cancer.

9. Packaging and Labeling

Hamburger packaging containers play a very important role in maintaining the quality, safety, shelf life and attractiveness of the product. These containers must comply with Iranian national standards. Hamburgers should be packaged in appropriate and permitted food-grade packaging with the approval and approval of the legal and competent authorities in Iran. The following information must be included on the label and packaging for marking:

Name and type of product - Percentage of meat used - Name and address of the production unit and its trademark - Date of production - Expiration

date - Storage conditions and consumption conditions (Iranian National Standardization Organization 2304, 2016).

10. Histological Characteristics

The history of histology dates back to the study of the microscopic structure of tissues and organs and has evolved over the centuries. The science has developed significantly with the development of the microscope and tissue preparation techniques. In the early 17th century, scientists such as Malpighi and Grew studied plant structures using early microscopes and recorded images of plant tissues (National Standard of Iran 6103, 2016; Asadi *et al.*, 2023; Yarvari *et al.*, 2025).

In the 19th century, with the development of the microscope and tissue preparation techniques, histology emerged as an independent science. During this period, scientists such as Johann Pürsinger and Marie Villiers developed techniques for staining tissues to view more detail. Histology also witnessed significant advances in the 20th century. New techniques such as the electron microscope made it possible to observe cellular and molecular structures with very high precision. Histology also became an applied field in medicine and biology with the advent of tissue engineering. And finally, in the 21st century, tissue engineering has made significant advances, with the aim of repairing and replacing damaged tissues using engineered cells and materials. hamburgers must be free from unauthorized organs and tissues of livestock and poultry. According to this standard, tissues of the thoracic and abdominal organs of livestock and poultry (such as heart, liver, trachea, lung, spleen, abomasum, intestines, esophagus, stratum corneum, gizzard, cloaca), urinary and reproductive organs (kidney, bladder, ovary, fallopian tubes, testicles and mammary glands), nervous tissues (spinal cord and brain) and lymphatic tissues (lymph nodes, tonsils, thymus, spleen and bursa of Fabricius), glandular tissues (such as salivary glands), tongue, skin, peritoneal fat, tail, bone tissues and cartilage are considered as unauthorized tissues in the production of meat products (National Standard of Iran 6103, 2016).

11. Storage Conditions

The storage conditions of hamburgers are very important for maintaining safety, quality and preventing spoilage. Failure to comply with these conditions can lead to the growth of dangerous bacteria such as salmonella and poisoning. The storage temperature of frozen raw hamburgers is minus 18 degrees Celsius and the cold chain should not be interrupted. Signs of spoilage of hamburgers include a pungent, sour or rotten smell, a change in color to gray, green or brown,

and the presence of slime on the surface (Iranian National Standardization Organization 2304, 2016).

12. Difference Between Iranian Standards and International Standards

The Iranian standard is more stringent in some cases than many international standards (such as Codex Alimentarius), especially in the field of fat percentage and restrictions on the use of additives.

13. Challenges in Implementing the Standard

Including insufficient supervision of small production units, differences in quality in different seasons, increased smuggling of meat and non-standard products, and lack of consumer awareness of the characteristics of a standard hamburger.

14. Recommendations for Consumers

Buy from reputable centers.
Pay attention to labeling and expiration date.
Pay attention to uniform texture and absence of heterogeneous parts.
Pay attention to the standard mark on the product.

15. Comparison of Iranian Standards with other Countries

The hamburger standard in Iran is more stringent than many countries in terms of restrictions on chemical additives and some quality parameters, but it needs to be developed in terms of information transparency, traceability system, and standards beyond safety. The best solution is to maintain the advantages of the current standard (such as restrictions on chemicals) and strengthen the weaknesses using international experiences. Standardization is a dynamic process and must be updated according to scientific advances, changing consumer tastes, and international trade requirements.

16. Conclusion

Compliance with the hamburger standard not only ensures consumer health, but also helps develop the country's food industry and increase public trust. Continuous supervision by competent authorities and informing consumers are the main pillars of the successful implementation of this standard. In Iran, special attention should be paid to the challenges and frauds and the issue of smuggling, and also, unauthorized additives and preservatives, and especially unauthorized textures in hamburger products, should be monitored

better and more. In the field of exports to Muslim countries in the Persian Gulf and in the issue of halal branding, the meat used should be further refined so that sanitary conditions and religious slaughtering and separation of forbidden components are carried out accurately.

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