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ORIGINAL ARTICLE

Protective Effects of Hydroalcoholic Extract of *Nigella Sativa* on Enzymatic Activity and Kidney Histopathology in Mice Infected with *Plasmodium Berghei*

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Abstract

Nigella sativa is a medicinal plant traditionally used for the treatment of various diseases. The presence of essential fatty acids in *Nigella sativa* has contributed to its anticancer properties and its effectiveness in promoting skin health. This study aimed to evaluate the protective effect of the hydroalcoholic extract of *Nigella sativa* on enzymatic activity and renal histopathology in mice infected with *Plasmodium berghei*. A total of 35 adult male mice, were divided into 7 groups. The mice were infected with *P. berghei* to induce malaria. After confirmation of infection, the mice were treated with *Nigella sativa* extract for 4 consecutive days. At the end of the treatment period, blood samples were collected to measure serum levels of urea and creatinine. Additionally, kidney tissues were subjected to histopathological examination. The results demonstrated that the *Nigella sativa* extract at a dose of 400 mg/kg reduced serum levels of urea and creatinine compared to the negative control group (PBS). Histopathological assessment of the kidney tissues revealed no significant morphological differences between the control and treated groups. Furthermore, the lumen and epithelial lining of the renal convoluted tubules in the extract-treated group appeared similar to those in the negative control group, with no statistically significant changes observed ($p>0.05$). Based on these findings, further long-term studies are recommended to investigate the potential of *Nigella sativa* extract in reducing elevated urea and creatinine levels in various pathological conditions. This extract may serve as an effective agent in managing renal dysfunction associated with different diseases.

1. Introduction

Malaria is one of the most common and deadly parasitic diseases worldwide, particularly in tropical and subtropical regions. In experimental models, *Plasmodium berghei* is the primary parasite used to induce malaria in mice, where it causes severe and often fatal infections. Due to its high pathogenicity in rodents, *P. berghei* is widely employed as a model organism for studying malaria pathogenesis and potential treatments *in vivo* (Hajialiani et al., 2021; Elmi et al., 2019).

Current treatments for malaria include a range of antimalarial drugs such as artemisinin, primaquine, chloroquine, mefloquine, and lumefantrine. However,

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in recent years, the emergence of drug-resistant strains-especially resistance to chloroquine-has become a major public health concern in many developing countries (Elmi et al., 2020; Wicht et al., 2020). Moreover, the side effects associated with antimalarial drugs and the increasing resistance of parasites to these therapies have significantly limited their effectiveness and sustainability. As a result, there is an urgent need to explore alternative therapeutic strategies for malaria management.

Malaria infection can cause severe damage to various internal organs, including the kidneys. Renal failure is one of the most serious and life-threatening complications of severe malaria, emphasizing the importance of investigating potential protective interventions against malaria-induced renal injury (Mishra and Das, 2008; Das, 2022).

Iran's diverse geography and climate provide a rich habitat for various medicinal plants, including *Nigella sativa* (black seed), a flowering plant from the Ranunculaceae family with pale blue to deep blue flowers. Traditionally, *Nigella sativa* has been used to treat numerous conditions, including bronchitis, asthma, rheumatism, and various skin disorders.

The seeds of *Nigella sativa* contain a wide range of bioactive compounds, including volatile and fixed oils, proteins, alkaloids, phenolic compounds, flavonoids, saturated and unsaturated fatty acids, terpenoids, and saponins (Shafodino et al., 2022; Alberts et al., 2024). The plant is known for its diverse pharmacological properties, such as antioxidant, anti-inflammatory, anticancer, analgesic, antimicrobial, and nephroprotective effects.

Given the rich therapeutic potential of *Nigella sativa* and the lack of studies investigating its protective effects on enzymatic activity and kidney tissue in malaria-infected individuals, this study was designed to evaluate, for the first time, the protective role of the hydroalcoholic extract of *Nigella sativa* seeds on renal histopathology and biochemical parameters in mice infected with *Plasmodium berghei*.

2. Materials and Methods

2.1. Extraction of Nigella Sativa

Fresh and pure *Nigella sativa* seeds were purchased and prepared for extraction. The seeds were first ground into a fine powder using an electric grinder. The resulting powder was then soaked in methanol for 72 hours (Khanabadi et al., 2022). After the soaking period, the mixture was filtered, and centrifugation was performed to eliminate any remaining suspended particles. The supernatant was separated, and the solution was concentrated using a rotary evaporator. Finally, the concentrated extract was freeze-dried (lyophilized) to obtain a dry powder. Various concentrations of the extract were prepared from this powder for experimental use.

2.2. Animal Preparation and Grouping

In this experiment, 35 adult male Syrian mice with an average body weight of 23±2 grams were used. The mice were acclimatized to the laboratory environment for one week prior to the experiment. Animals were housed in individual cages under controlled conditions (temperature: 22-26°C, relative humidity: 40–70%, and a 12-hour light/dark cycle) with ad libitum access to food and water. The mice were then randomly divided into seven groups.

All groups except the seventh were infected with *Plasmodium berghei* to induce malaria. After confirmation of infection, the mice received treatment with *Nigella sativa* extract for four consecutive days (Elmi et al., 2022). The dosage of the extract was determined based on established therapeutic limits. Group 1 served as the negative control (PBS), Group 2 as the positive control (chloroquine), and Groups 3 through 6 received 50, 100, 200, and 400 mg/kg of *Nigella sativa* extract, respectively. Group 7 included healthy, untreated mice and was used to monitor baseline mortality during the study.

2.3. Sample Collection

At the end of the treatment period, the mice were anesthetized, and blood was collected directly from the heart. Following blood collection, the animals were euthanized by cervical dislocation. Blood samples were centrifuged at 3000 rpm for 5 minutes to separate the serum, which was then stored at –80°C for biochemical analysis. Kidney tissues were preserved in 10% formalin for histopathological evaluation.

2.4. Histological Section Preparation

Kidney samples were fixed in 10% buffered formalin at room temperature for histomorphometric analysis. The tissues were dehydrated through graded ethanol concentrations (70%, 80%, 90%), cleared in xylene, and embedded in molten paraffin for 4 hours. Paraffin blocks were sectioned using a microtome to obtain 5 μm thick slides, which were then stained with hematoxylin and eosin (H&E) (Elmi et al., 2019).

2.5. Histometric Analysis of Kidney Samples

Histological sections of the kidneys were examined for Bowman's capsule integrity, glomerular network structure, degeneration of proximal and distal tubules, vascular congestion, and interstitial edema. Histopathological alterations were scored on a scale from 0 to 3: 0 = no change, 1=mild changes, 2=moderate changes, and 3=severe changes.

2.6. Hematological and Biochemical Analyses

Biochemical parameters, including serum urea and creatinine levels, were measured using spectrophotometry, following the instructions provided with the commercial assay kits. Serum biochemical analyses were conducted using an automated analyzer.

2.7. Statistical Analysis

All data were analyzed using SPSS software version 22. One-way analysis of variance (ANOVA) and the Kruskal-Wallis test were employed for statistical comparisons. Data were presented as mean ± standard deviation (SD), and a p-value of less than 0.05 was considered statistically significant

3. Results

3.1. Body Weight of Mice

The analysis of body weight among different groups showed that the average weight of mice in the negative control group was 26.8 g, in the positive control group was 26.2 g, and in the group treated with 400 mg/kg of *Nigella sativa* extract was 27 g. According to the results, there was no statistically significant difference in body weight among the groups ($p>0.05$). These findings suggest that treatment with *Nigella sativa* extract had no significant effect on the body weight of mice (Fig. 1).

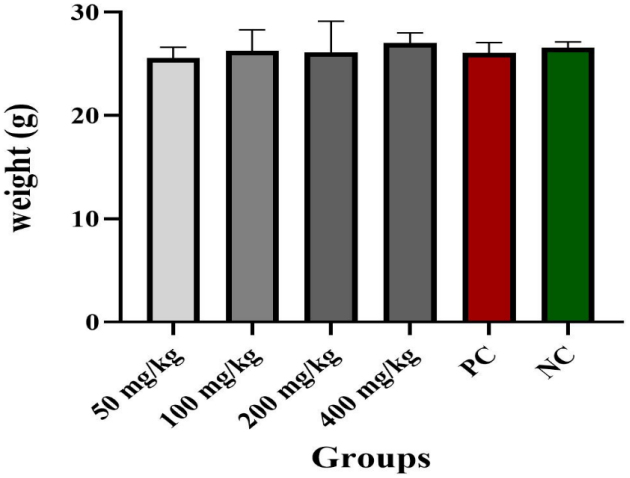

Fig. 1. Comparison of body weight in malaria-infected mice following treatment in negative control (NC), positive control (PC), and different doses of *Nigella sativa* extract.

3.2. Serum Urea Levels

Treatment with *Nigella sativa* extract at doses of 100, 200, and 400 mg/kg significantly reduced serum urea levels compared to the negative control group receiving PBS ($p<0.05$). The most prominent reduction was observed in the group treated with 400 mg/kg of the extract, where serum urea levels decreased from 29.06 mg/dL to 26.18 mg/dL (Fig. 2). This significant decrease in serum urea indicates a nephroprotective effect of the extract in improving kidney function and reducing uremic toxicity in malaria-infected mice.

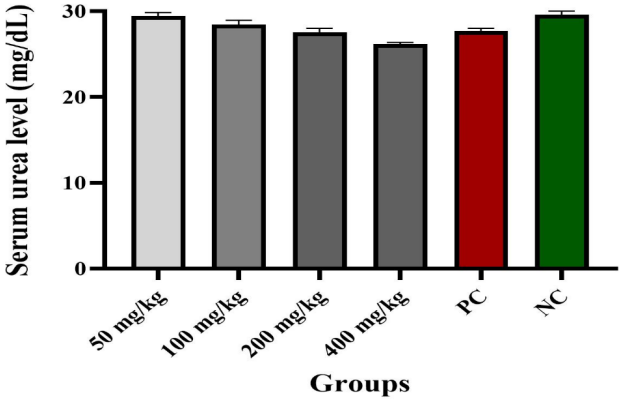

Fig. 2. . Serum urea levels in negative control (NC), positive control (PC), and groups treated with various doses of *Nigella sativa* extract.

3.3. Serum Creatinine Levels

Treatment with *Nigella sativa* extract at doses of 50, 100, and 200 mg/kg did not significantly reduce serum creatinine levels compared to the negative control group ($p>0.05$). However, the most notable decrease was observed at the dose of 400 mg/kg, where creatinine levels declined from 0.54 mg/dL to 0.42 mg/dL (Fig. 3).

3.4. Kidney Histopathology

Histological evaluations revealed no significant differences in renal tissue structure between the control and treated groups ($p>0.05$). The morphology of renal tubule lumens and epithelial lining in the extract-treated group was similar to that of the negative control, and no notable histological alterations were observed (Fig. 4).

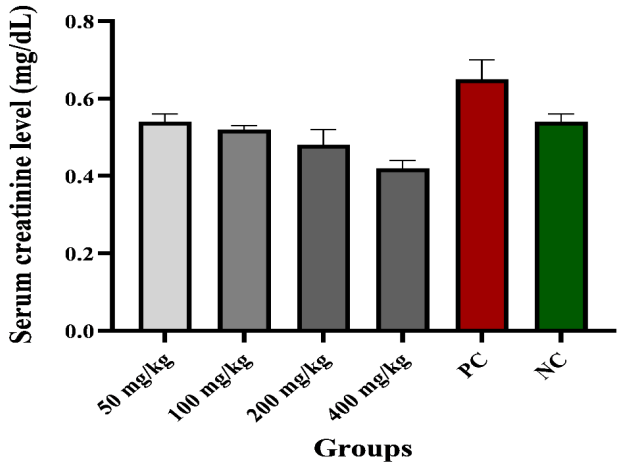

Fig. 3. Serum creatinine levels in negative control (NC), positive control (PC), and groups treated with various doses of *Nigella sativa* extract.

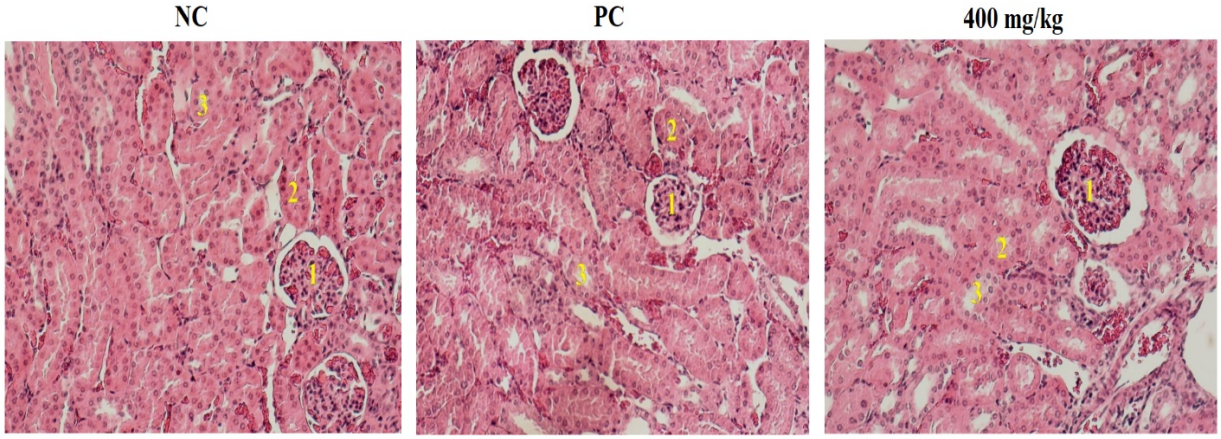

Fig. 4. Histopathological analysis of kidney tissue in negative control (NC), positive control (PC), and the group treated with 400 mg/kg of *Nigella sativa* extract. Labels 1, 2, and 3 indicate the renal corpuscle, proximal convoluted tubule, and distal convoluted tubule, respectively.

4. Discussion

Malaria, caused by *Plasmodium* parasites, remains one of the most detrimental parasitic diseases affecting human health, particularly in tropical regions. Due to the growing challenges of drug resistance and the adverse effects of conventional antimalarial drugs, research into alternative treatments has become increasingly important. Among these alternatives, medicinal plants have gained significant attention in recent years. In this study, the protective effects of the hydroalcoholic extract of *Nigella sativa* seeds on enzymatic activity and kidney tissue in mice infected with *Plasmodium berghei*-induced malaria were evaluated.

The results of the present study showed that administration of *Nigella sativa* extract at various doses did not have a significant effect on body weight. This finding is consistent with previous studies. For instance, Ali et al. (2012) reported that oral administration of *Nigella sativa* extract over a short period did not alter the body weight of rats, suggesting that the bioactive compounds of *Nigella sativa* may not significantly affect general metabolism or appetite within a similar timeframe (Ali et al., 2003).

However, other studies have reported either an increase or decrease in body weight, often in a dose-dependent manner and over longer periods. For example, a study by Bashir *et al.* (2023) demonstrated that *Nigella sativa* extract at various doses (100–400 mg/kg) improved conditions such as diet-induced obesity, non-alcoholic fatty liver disease (NAFLD), hyperlipidemia, and diabetic nephropathy compared to metformin (250 mg/kg) (Bashir et al., 2023). Notably, the 200 mg/kg dose significantly alleviated metabolic disorders induced by a high-fat diet, which is in line with some of the metabolic effects observed in the present study.

A key finding of this study was the significant reduction in serum urea levels, particularly at the 400 mg/kg dose of *Nigella sativa* extract. This effect may reflect the nephroprotective properties of the extract. Similar results were reported by Al-Ghamdi *et al.* (2003), where *Nigella sativa* conferred renal protection against drug-induced nephrotoxicity by lowering nitrogenous markers such as urea and blood urea nitrogen (BUN) (Al-Ghamdi, 2001).

From a pharmacological perspective, the active compound thymoquinone found in *Nigella sativa* possesses strong antioxidant and anti-inflammatory properties (Bordoni et al., 2019). These properties may help reduce oxidative stress in renal tubular cells, thereby improving renal excretory function. In animal models with drug- or infection-induced renal injury, thymoquinone has been shown to reduce urea levels by inhibiting inflammatory pathways such as NF-κB (Kordestani et al., 2020; Alkis et al., 2021).

In contrast to urea, a reduction in serum creatinine was observed only at the highest dose (400 mg/kg). This may suggest that creatinine is less sensitive to mild renal functional changes than urea, or that the extract did not exert a complete protective effect on glomerular structure and filtration rate.

Supporting this, Mehrdad et al. reported that ginger extract significantly reduced BUN levels in treated mice, with a non-linear relationship to the administered dose (Mehrdad et al., 2007). However, only minimal changes in serum creatinine levels were observed compared to the control group.

Histopathological findings in this study demonstrated preserved kidney architecture in the extract-treated groups, with no significant pathological alterations observed. These results are consistent with the biochemical findings, as the reduction in serum urea in the absence of severe histological damage may indicate early-stage kidney protection under disease conditions.

5. Conclusion

The results of this study indicate that *Nigella sativa* seed extract, particularly at a dose of 400 mg/kg, exerts protective effects against malaria-induced renal damage and may be considered as a potential adjunct therapy for patients with this condition. However, further studies are warranted to evaluate the long-term effects of this extract and to elucidate the precise mechanisms underlying its influence on kidney function and other organs.

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REVIEW ARTICLE

Usage of the Cell Dissociation Technique in Reproductive Studies; Study on Mechanical Cell Dissociation in Male Mice in Maturity Course

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Abstract

Cell dissociation techniques are one of the main methods for separating different cells from various tissues. These techniques consist of two main approaches: chemical and mechanical separation. Each technique has its own advantages and disadvantages, and the most suitable method should be selected. In the chemical method, specific enzymes must be used. In the mechanical method, cells are separated by mechanical force. Normally, in most research studies, the chemical method using specific enzymes is more commonly applied. In this study, 15 mice were used during the maturity phase and were grown under standard conditions. These mice were operated on after 32 days, and the testes were extracted. The cells were dissociated using the mechanical method. Finally, the samples were stained with Giemsa and examined. The advantages of these techniques include the separation of undamaged cells, which were observed in both methods. However, the main focus of this paper was mechanical cell dissociation, which did not damage the primary cells. Moreover, this method can be used for chromatin studies by counting each cell type and separating them to investigate testicular functions. Therefore, using mechanical cell dissociation for the seminiferous tubule wall could be a useful and economical option as a low-cost method for comparative studies involving cells in testicular tissue. The findings suggest that mechanical dissociation provides an efficient and low-cost method for isolating intact testicular cells, making it suitable for histological and functional studies of seminiferous tubules.

1. Introduction

At this time, to understand the complex mechanisms of spermatogenesis, we study all the changes in surface antigens on the cells. Studying the expression patterns of various genes will molecularly evaluate the process of spermatogenesis, and these studies identify key factors in the control of spermatogenesis as well as other cytological aspects. Molecular studies require certain conditions, such as the isolation and identification of spermatogenic cells from testicular tissue, which is the main tissue examined here (Schneider *et al.*, 2015).

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According to all data, the first report on cell dissociation and identification of spermatogenic cells was published in 1977 (Bellvé *et al.*, 1977). They succeeded in obtaining a pure population of these cells from the testicular tissue of immature mice.

In this process of separating tissue cells from each other, they used mechanical methods and enzymatic digestion, and then applied SVUG (sedimentation velocity at unit gravity) as an appropriate technique to successfully extract pure populations of all six types of spermatogenic cells from the tissue during the growth and development of the testis in Balb/c mice (Schneider *et al.*, 2015; Zhang *et al.*, 2016). During the isolation phase of various populations of spermatogenic cells obtained from testicular tissue, they began using mechanical methods.

There are two methods for cell dissociation related to this function (Lima *et al.*, 2017).

1) Chemical method

2) Mechanical method

In the chemical method, enzymes like trypsin and collagenase are used for cell dissociation. In the mechanical method, special equipment with a specific technique called "sedimentation velocity at unit gravity" is used (Lima *et al.*, 2016; Tepperman *et al.*, 1975).

In addition to this, as a simpler and easier method, a tool such as scissors can be used to cut soft tissue such as the testes to access the inner cells such as spermatozoa (Montanari *et al.*, 2022; Goldberg, 2015).

2. Spermatogenic Cells and All Methods for Cell Dissociation Process

Spermatogenic cells that are present in cell cultures will be characterized according to their shape. Different elements include size, diameter, location of the nucleus, flagella cells, presence of acrosomal structures (e.g., haploid testicular cells), and their own staining quality (Schneider *et al.*, 2015; Zhang *et al.*, 2016). In the mechanical method, special equipment with a specific technique called "sedimentation velocity at unit gravity" is used. Alternatively, for soft tissues such as testes, which we are working with here, simple methods such as cutting with a tool like scissors can be used to isolate interior cells such as sperms (Montanari *et al.*, 2022).

Chemical dissociation methods using a variety of enzymes, such as different types of collagenases and trypsin, can be expensive. Studies have shown that cells obtained by enzymatic dissociation using enzymes like trypsin or pronase during trypsinization show limited proliferation in serum-free medium after washing with saline.

It is also possible that trypsin and pronase bind to the surface of cells and alter the surface structure, making it different. This function affects cell adhesion. Once this surface is altered, the cells may take some time to regain their ability to adhere to the dish and target, which can be observed when transferred to another container.

At this time, it is necessary for the cell surface to be remodeled or repaired to resolve the problems (Montanari *et al.*, 2022). Two enzymes that are useful and can be quite powerful for chemical dissociation are collagenases and trypsin.

3. Collagenase

This enzyme may be divided into two kinds that are primarily and totally based on its presence in distinct pathways:

1) Endogenous human collagenase

2) Pharmaceutical collagenase

The human body naturally produces some collagenase inside tissues such as gums, epithelial tissue, and synovial surfaces. These various types of collagenases play an essential role in breaking down collagen within the body during intra-distal processes, and this kind is called endogenous collagenase (Schol *et al.*, 2024).

4. Different Types of Collagenases and their Applications and Functions of Collagenase

These types and functions exist in numerous forms, which are summarized in Table 1 (Worthington, 1988). Some of the more general types include Collagenase I-V (Wu *et al.*, 2023).

4.1. Collagenase I

This enzyme is used for many tissues such as epithelium, lung, fat, and the isolation of adrenal tissue cells. It is appropriate for digesting tissues and converting them to a unicellular form. so, it is useful for separating mammalian cells (Worthington, 1988; Wu *et al.*, 2024).

4.2. Collagenase II

This enzyme is a suitable and effective agent for tissues such as liver, bone, thyroid, heart, and salivary glands (Worthington, 1988; Shajib *et al.*, 2022).

4.3. Collagenase III

Collagenase type III is a bacterial enzyme capable of degrading collagen types I, II, and III, particularly in dense connective tissues. It enables efficient cell isolation while preserving membrane integrity, making it

suitable for tissue engineering and cell culture applications (Wu *et al.*, 2023).

4.4. Collagenase IV

This kind of enzyme consists of at least seven protease additive factors with molecular weights ranging from 68 to 103 kDa.

In addition to all these properties, this enzyme can also digest various forms of tissue (Wu *et al.*, 2023).

4.5. Collagenase V

This enzyme contains at least seven protease components with molecular weights ranging from 68 to 103 kDa.

It may be used to separate pancreatic islets from all parts of the main tissue. It is also possible to isolate unique cells with this enzyme.

When an organization wants to digest and separate hard tissue consisting of connective tissue or collagen, cell dissociation with trypsin is much less effective. Therefore, collagenase may be used for more complete and specific separation.

Interstitial digestion of cells through collagenase is possible and has minimal impact on epithelial cells. Consequently, it is a highly appropriate enzyme for isolating and digesting fibrous tissue, epithelial cells, cancerous tissue, and collagen fibers. It allows separation without harming or damaging the cells (Worthington, 1988; Alipour *et al.*, 2016).

5. The Mechanical Approach

This method is a way to carry out cell dissociation without using any enzymes or chemical materials. For example, in the article by C. Broadley and colleagues, cells were separated by cutting the sample into small parts and placing them in a centrifuge at high speeds (Schneider *et al.*, 2015; Shetab-Boushehri and Shetab-Boushehri, 2024; Broadley *et al.*, 1994). In other articles, such as "A Plasma-Deposited Surface for Cell Sheet Engineering: Advantages over Mechanical Cell Dissociation", Heather E. and her colleagues compared these techniques and concluded that there is a relationship with the mechanism of cell death.

These results show that mechanical cell dissociation contrasts with apoptotic mechanisms, because it causes lipid membrane rupture, leading to the discharge of intracellular contents and contributing to irritation and swelling.

Therefore, cells obtained by the mechanical method are used in studies where destructive mediators are present. In sequence analysis studies, it is common to use enzymatic methods and cells obtained through enzymatic dissociation (Canavan *et al.*, 2006).

6. Material and Methods

The main goal of this study is to become familiar with different cell dissociation techniques that are used in various studies, such as reproductive research. In addition, it aims to examine mechanical cell dissociation as an economical technique for studying different testicular cells at minimal cost.

The main study was performed on 15 male mice, all of which were kept under standard temperature and nutritional conditions. In these experiments, mice were euthanized after 32 days, and according to the image in Fig. 1, their testes were successfully extracted (Fig. 1; Abbasi-Malati *et al.*, 2018). In this process, we weighed the mice before surgery and weighed the right testis before and after fixation. In addition, the measurements of length, width, and diameter were recorded and presented in charts.

Following all procedures, after collecting the right testicular tissue, each testis was placed in 1 ml of physiological serum and cut into small pieces with sharp scissors, as small as possible, to release spermatozoa cells from the seminiferous tubules.

Table 1
Classification of collagenases and their substrates (Worthington, 1988; Alipour *et al.*, 2016).

Row	Enzyme	Matrix metalloproteinase	Group	Substrate
1	Collagenase 1	MMP-1	Collagenases	Collagens 1, 3, 7, 8, 10, gelatin, L- selectin, interleukin-1, entactin, ovostatin, MMP-2, MMP-9, proteoglycans, aggrecan
2	Collagenase 2/Neutrophil collagenase	MMP-8	Collagenases	Collagens 1, 3, 5, 7, 8, 10, fibronectin, gelatin, aggrecan
3	Collagenases 3	MMP-13	Collagenases	Collagens 1, 4, 9, 10, 14, fibronectin, MMP-9, gelatin, plasminogen, aggrecan, perlecan osteonectin
4	Collagenases 4	MMP-18	Collagenases	Type I collagen

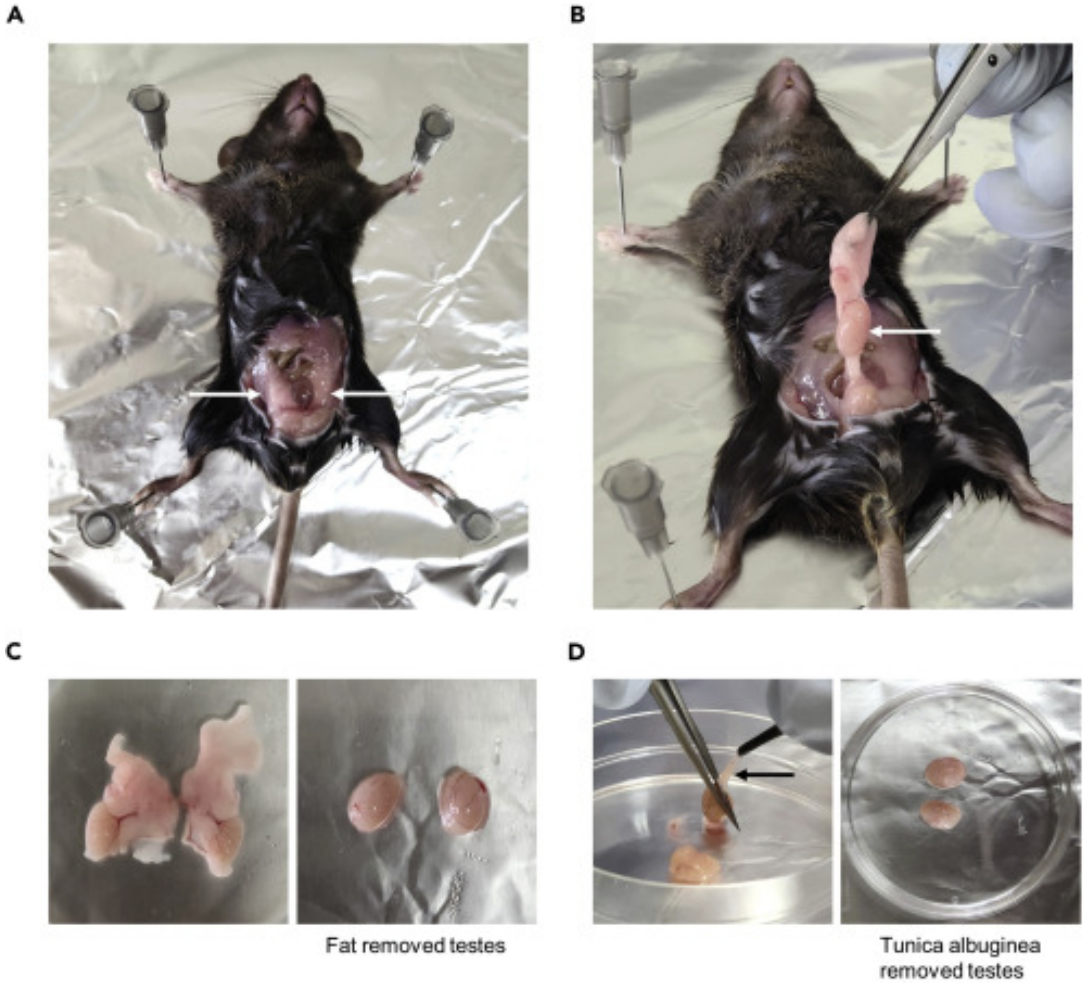

Fig. 1. Mice surgery for extracting testes tissue and making it ready for mechanical cell dissociation (Abbasi-Malati *et al.*, 2018).

After that, according to our method for investigating these cells, 2–3 drops of the resulting suspension were taken from the main sample and placed on a gelatin-coated slide to achieve the desired spread without drawing another slide over it.

The selected slides were placed in a laboratory environment to dry, and after drying, pure methanol was added to cover the entire surface of the slides. The methanol remained on the sample until only a thin layer of moisture was visible on each slide, indicating the staining time.

The samples were stained with Giemsa stain for 15 minutes (Othman *et al.*, 2023; Tsouloufi *et al.*, 2020). After staining, the samples were first rinsed with distilled water, then placed in xylene, and finally dried in the laboratory environment (Schneider *et al.*, 2015).

After drying, a larger cover slip was attached to the slide using Entellan glue to preserve the sample under standard conditions. The results of cell dissociation from mouse testicular tissue were studied under a microscope.

The mice used in these experiments belonged to the control group and did not receive any treatment.

7. Results

According to the experiment and all procedures performed, mechanical cell dissociation of spermatogenic cells from mouse testes was successful, showing different cell types in the prepared slide samples. In this way, different cell types were clearly observed and easily distinguished from each other.

Finally, images of the prepared samples (Fig. 2) are provided here. Different shapes of spermatogenic cells were captured and can be seen in the images.

The analysis of testis size before and after the fixation process was conducted to determine whether the mechanical procedure caused any structural damage to the tissue. Based on the recorded measurements and statistical analysis, the p-value was greater than 0.05, indicating no significant difference in testis dimensions ($P>0.05$).

According to all date of weights and measurements, we have some charts, and after studying all charts, we can see that the mice are in the same condition in terms of testis and size information. You can see the charts below (Figs. 3, 4, 5, 6).

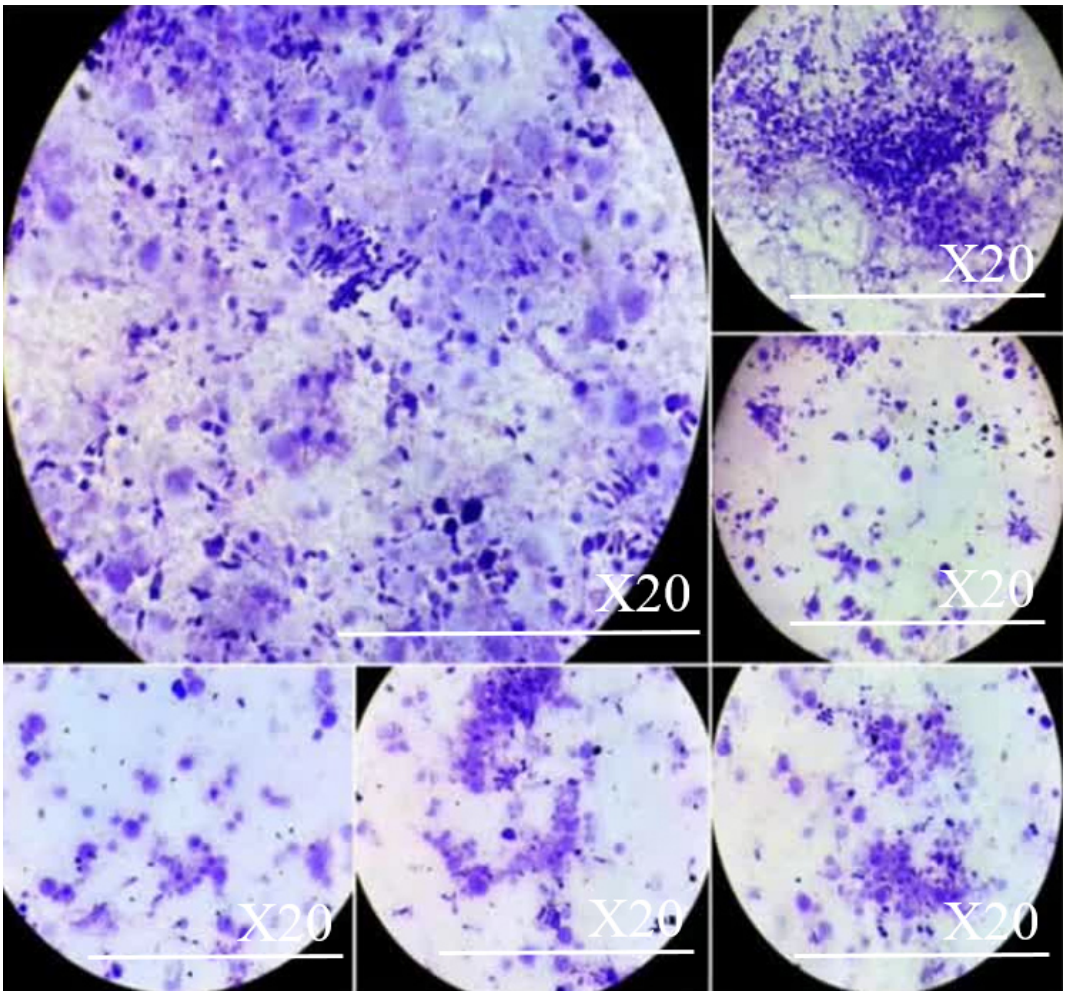

Fig. 2. Different types of sperm in different steps, with different shapes. These cells were seen in mechanical cell dissociation samples, they were derived from 15 mice without any treatment.

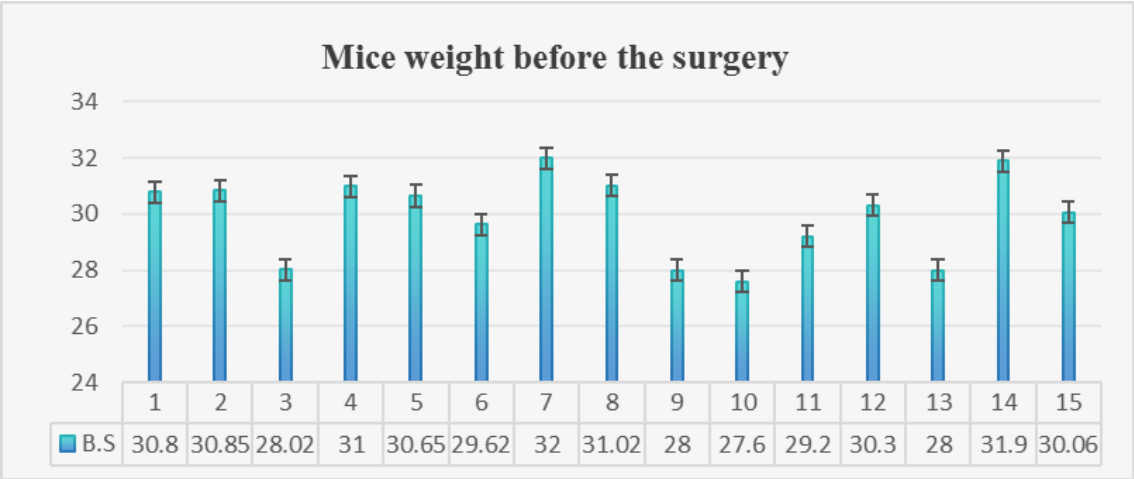

Fig. 3. Mice weight before surgery at 32 days old. All the mice were in the same condition, and their weights were nearly the same, and similarity was observed. "B.S." in the chart is the main group before the surgery process.

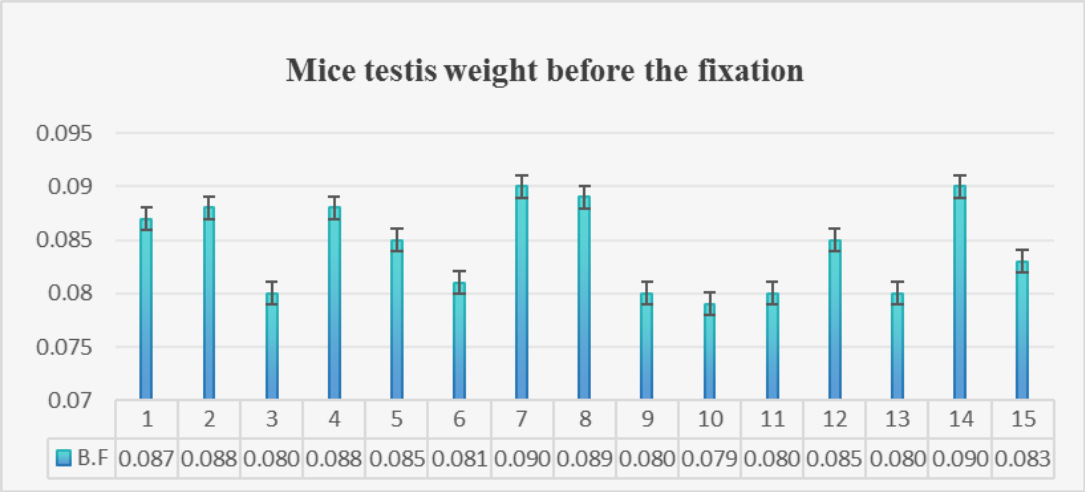

Fig. 4. Mice testis weight before fixation was more than after fixation. "B.F." in the chart is the main group before the fixation process.

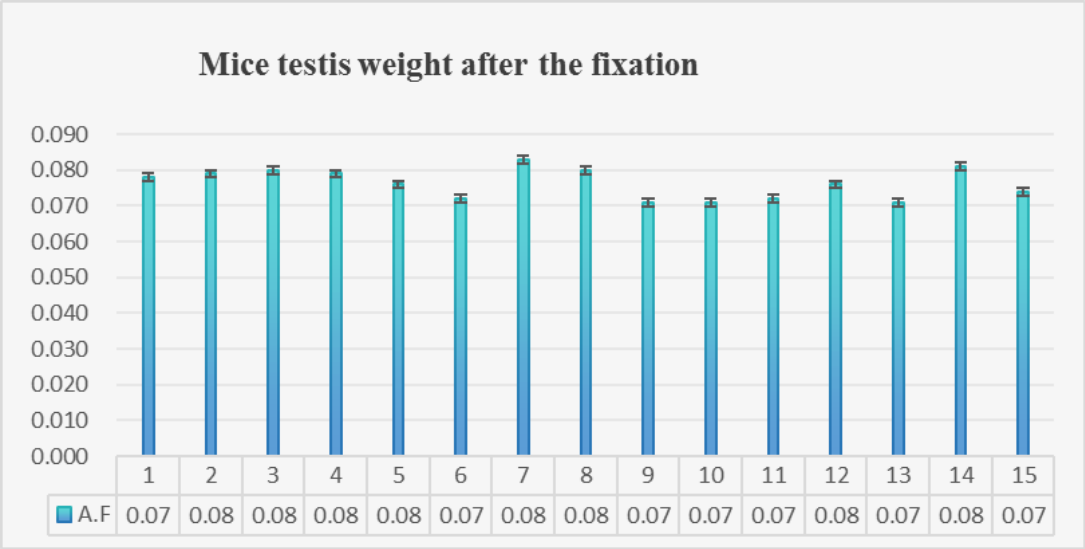

Fig. 5. Mice testis weight after fixation was less than before fixation. "A.F." in the chart is the main group after the fixation process.

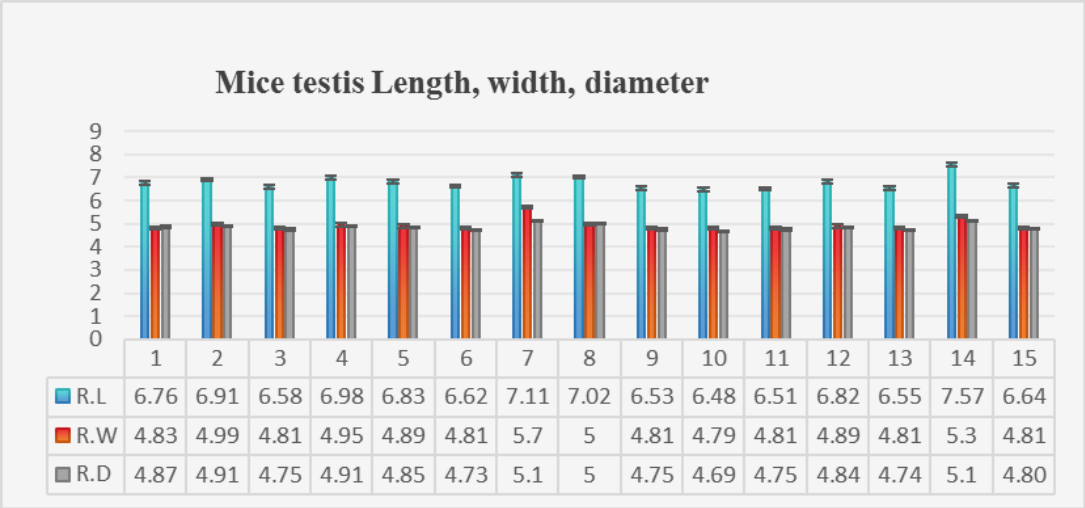

Fig. 6. In this chart, the first column represents the right testis length in mice and is labeled as "R.L.". The second column shows the right testis width, labeled as "R.W.", and the third column indicates the right testis diameter in mice, labeled as "R.D.".

8. Discussion and Conclusion

In this paper, according to the introduction of the cell dissociation methods for studying different cell types of the seminiferous tubule wall of testis tissue, it can be chosen as an ideal and economical method for histological techniques. Therefore, this method, as demonstrated in this article, can be used to isolate and separate different cells of main tissues for different purposes and targets, such as reproductive studies. This allows for the achievement of appropriate and efficient results at a low cost.

According to the results of this experiment and study, different cell types were observed within the field of view, and they were countable.

so, it is possible to study the causes of infertility by counting the gametes in mice that were treated with different dietary or non-dietary substances. This may help to study and determine whether the cause of infertility is due to a reduction in the number of gametes or because of changes in their normal morphology. Also, it is important to know the ages of mice at which these substances have significant effects on the gamete cells.

In this study, mechanical cell dissociation was evaluated as a practical and economical method for isolating spermatogenic cells from testicular tissue. The analysis of testis size before and after the fixation process was conducted to determine whether the mechanical procedure caused any structural damage to the tissue. Based on the recorded measurements and statistical analysis, the P-value was greater than 0.05, indicating no significant difference in testis dimensions. This suggests that the mechanical dissociation method preserves tissue integrity and does not induce measurable damage to the seminiferous tubules (Abbasi-Malati *et al.*, 2018).

The absence of significant morphological changes following fixation supports the reliability of this approach, particularly in studies where enzymatic reagents are either unavailable or undesirable due to cost, accessibility, or potential chemical interference with cellular structures. Unlike enzymatic methods, which may alter cell surface proteins or affect downstream molecular analyses, mechanical dissociation offers a non-invasive alternative that maintains the native architecture of the cells.

Furthermore, the ability to isolate intact and countable spermatogenic cells using this method opens new avenues for reproductive research. For example, in studies investigating infertility, quantifying gametes and assessing their morphology in response to dietary or pharmacological interventions is essential. The mechanical method enables researchers to perform such analyses without introducing chemical artifacts, making it especially valuable in toxicological, developmental, and histopathological contexts (Othman *et al.*, 2023).

Additionally, this technique may be particularly beneficial in resource-limited settings, where access to specialized enzymes such as trypsin or collagenase is restricted. The simplicity of the protocol using basic laboratory tools such as scissors and physiological serum makes it adaptable for routine use in both academic and clinical laboratories. Its cost-effectiveness and reproducibility further enhance its appeal for large-scale studies and comparative analyses (Tsouloufi *et al.*, 2020).

In conclusion, mechanical cell dissociation represents a viable and efficient method for testicular tissue processing. It ensures minimal tissue disruption, preserves cellular morphology, and provides a reliable platform for downstream applications such as cell counting, functional assays. Given its advantages, this method can be considered a suitable alternative to enzymatic dissociation, especially when the preservation of tissue integrity and reduction of experimental costs are prioritized.

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REVIEW ARTICLE

A Comprehensive Review of Advanced Modern Treatments, Strategies, and Techniques for Accelerating Wound Healing

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Abstract

Skin wounds are common events and may occur in different situations for each person, but it's better to start the skin repairing process as soon as possible. So, the healing happens in the best time with the minimum scar and skin tissue damages. Recent developments in wound management have introduced the advanced therapeutic modalities for repairing the tissue, and each of the approaches contributes to enhancing tissue regeneration. Therefore, according to all developments in methods for increasing the speed of healing, it can be seen in skin wound healing processes too. So, it's necessary to know more about wound healing and different modern treatments and techniques for accelerating wound healing, especially in skin injuries. This review focuses on exploring the wound healing process and different advanced and modern treatments to introduce and review all these items together. It is useful for anyone who needs general data about advanced wound healing techniques.

1. Introduction

In different situations when an injury happens, we have to care for the wound and find a way for accelerating the wound healing in the oral and facial wound healing after traumatic injuries in different situations such as car accidents, knife wounds, lacerations, and surgeries, or wounds heal through hemostasis, inflammation, proliferation, and remodeling phases of wound healing, involving multiple cells and cytokines in the oral and maxillofacial regions. The healing process and all of its stages are explained below for more information and studying with the best data.

2. Stages of Wound Healing

According to all data that have been given by physiologists about the physiological changes that are observed in obesity, it is possible that they directly influence the wound healing process. So, for understanding possible interaction mechanisms, it requires familiarity with the wound healing process under normal physiological conditions. In other words, hemostasis, the first step in wound healing, occurs when endothelial cells are damaged. After hemostasis, the inflammatory phase begins with edema and an influx of inflammatory cells. The inflammatory phase is followed by proliferation and remodeling, the latter of which can last for more than 2 years (Gurtner *et al.*, 2008).

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2.1. Hemostasis

Hemostasis, the first step in wound healing, occurs through endothelial damage and can last from minutes to hours. Immediately after injury, vasoconstriction of the injured vessel occurs, and both the intrinsic and extrinsic coagulation cascades are activated by adjacent platelets and endothelial cells. The blood clot that forms is rich in platelets, collagen, fibronectin, and thrombin. It acts as a scaffold for neutrophils, monocytes, and other invading cells, triggering the release of local vasoconstrictors such as cytokines, growth factors, and serotonin (George Broughton *et al.*, 2006). After hemostasis, the release of histamine induces the migration of inflammatory cells to the injury site, and it is the beginning of the inflammatory phase.

2.2. Inflammatory Phase

The inflammatory phase typically begins shortly after the initial injury and hemostasis phase (Eming *et al.*, 2014), and this phase is important for the wound healing process as it marks the recruitment of the innate immune system. Within the first 48–96 hours after injury, monocytes are recruited from the surrounding tissues and transformed into macrophages (George Broughton *et al.*, 2006), and these activated macrophages are required for the transition from the inflammatory phase to the proliferative phase. In addition, macrophages also release vascular endothelial growth factor, fibroblast growth factor, and TNF-α to stimulate angiogenesis. At this time, the major cell type at this stage is neutrophil. These important cells are recruited to the injury site via IL-8 that was released from platelets during the degranulation process and secrete IL-1, TNF-α, and TGF-β at this time too (George Broughton *et al.*, 2006). After injury in skin, Toll-like receptors are expressed on host cells and activate two distinct signaling pathways: 1. the nuclear factor kappa beta signaling pathway and 2. the mitogen-activated protein kinase signaling pathway. Activation of these signaling pathways is a hallmark of the inflammatory phase (Landén *et al.*, 2016). Once the inflammatory phase subsides, the body enters the proliferative phase. The inflammatory phase can be the reason for healing and other skin issues. So, the healing will begin only when the inflammatory phase ends completely (Guo and DiPietro, 2010).

2.3. Proliferative Phase

During the proliferative phase, the body prioritizes the restoration of the local vascular network and re-epithelialization of the wound surface too (Landén *et al.*, 2016). When the keratinocytes begin to migrate from the edges of the wound bed, and the epithelial stem cells begin to proliferate in response to influenced chemical and mechanical signals from both anti-inflammatory and pro-inflammatory cells, it will lead to fibrosis. During the fibrogenesis, fibroblasts are activated and transform into myofibroblasts and secrete components of the extracellular matrix that promote the wound contraction and contribute to permanent scar formation (Zangooei and Jalili, 2013). These signals lead to the development of granulation tissue, which is primarily composed of collagen III, new blood vessels, and fibroblasts. Fibroblasts are the main cell type in granulation tissue and initiate the re-epithelialization in response to cytokines that were released by macrophages. In addition to IL-6, fibroblasts release keratinocyte growth factors 1 and 2, and these cytokines stimulate local keratinocyte migration, proliferation, and differentiation in the wound bed (George Broughton *et al.*, 2006). Studies have shown that collagen deposition, epithelialization, and angiogenesis are reduced in wounds lacking IL-6 (Lin *et al.*, 2003). The complex of the four stages of wound healing is important to consider, especially how these stages are affected by obesity.

2.4. Remodeling

During the maturation and remodeling phase, the wound reaches its maximum strength as it matures (Bowden *et al.*, 2016).

3. Process of Skin Wound Healing

The complex healing process of skin wounds involves various cellular and molecular processes. Wound healing is highly dependent on reactive oxygen species (ROS), and it is essential for the control of various processes such as inflammation, cell proliferation, angiogenesis, granulation, and extracellular matrix formation. Nevertheless, excess reactive oxygen species (ROS) caused by increased oxidative pressure can lead to delayed or failed wound healing. It is important to understand the function of reactive oxygen species (ROS) and develop biomaterials that efficiently scavenge ROS and improve the skin wound healing process. This study represents a thorough investigation of the role of reactive oxygen species (ROS) in the wound healing process and examines existing knowledge regarding biomaterials that were used for ROS removal. Additionally, this article describes various techniques and substances that can be used to treat skin wounds. The clinical application of improved biomaterials is also highlighted. In fact, it has the potential to bind to reactive oxygen species and promote skin repair. Its goal is to develop new strategies for the effective treatment of cutaneous wounds (Bedard and Krause, 2007; Roy *et al.*, 2006; Soneja *et al.*, 2005).

4. Difficult and Poor Wound Healing Formations

Chronic wounds can result in formations where one step of wound healing is impaired. The main characteristics of chronic wounds are a prolonged inflammatory phase, excessive neutrophil infiltration, persistent infection, and high MMP levels. In chronic wounds and ulcers, fibroblasts are phenotypically distinct and have reduced migration capacity compared to those in acute wounds (Brumberg *et al.*, 2021). Dysregulation of pro-inflammatory cytokines such as IL-1β and TNF prolongs the inflammatory phase and delays healing. It has been shown that IL-1β and TNF are increased in chronic wounds, leading to elevated MMP levels and excessive degradation of local ECM, impairing cell migration (Hesketh *et al.*, 2017; Krzyszczyk *et al.*, 2018). MMPs such as collagenase and gelatinase A and B have been demonstrated to be increased in body fluids of chronic wounds compared to acute wounds (Eming *et al.*, 2007). Additionally, insufficient angiogenesis may contribute to chronic wounds due to the lack of pro-angiogenic factors such as members of the VEGF family, caused by proteolysis and subsequent impairment of biological activity in the chronic wound microenvironment (Lauer *et al.*, 2000). Chronic wound development involves several cellular and signaling pathways and several molecular markers downstream of the Wnt signaling pathway. Activation of the β-catenin/c-myc pathway promotes the healing of injured tissues by inhibiting keratinocyte migration, altering their differentiation, and suppressing keratinization (Stojadinovic *et al.*, 2014; Stojadinovic *et al.*, 2005). In this way, EGF receptors are inhibited at the non-healing edges of keratinocytes (Brem *et al.*, 2007). Furthermore, in chronic wounds, TGFβ signaling is suppressed by downregulation of TGFβ receptors and attenuation of Smad signaling (Pastar *et al.*, 2010). Such cell signaling may serve as a target for rapid wound healing in chronic wounds.

5. Current Wound Healing Treatment Methods

- Dressings
- Negative Pressure Therapy
- Surgery
- Hyperbaric Oxygen Therapy

6. Wound Healing Dressing

A variety of cells and growth factors are involved in the wound healing process, and traditional dressings cannot control the release of drugs in response to the healing process. Intelligent wound dressings can selectively release some active ingredients in response to changes in temperature, light, magnetic fields, pH, enzyme levels, and ROS at the wound site. Additionally, the physical properties of the hydrogel's synthetic components and its 3D pore-like structure enable excellent drug loading capacity. Medicated hydrogels, compared with traditional wound dressings, can respond to the local environment by releasing active substances and can regulate different stages of wound healing. Hydrogel acts as a bio-dressing loaded with functional drugs in response to stimulation and can promote the drug release process (Qian *et al.*, 2020).

7. Hydrogels in Wound Healing

Hydrogels are a category of materials with widespread applications in the field of skin regeneration. These are polymeric structures that exist in three-dimensional forms and are formed by physical or chemical cross-linking of hydrophilic polymer chains (Koehler *et al.*, 2018). These can be synthesized using various techniques such as irradiation, freeze-thaw, and chemical processes. Hydrogels are called "reversible" or "physical" gels when their structural integrity is maintained by molecular entanglements or ionic compounds. Hydrogen bond gels are composed of covalent bonds and are called "permanent" or "chemical" gels. These networks can potentially undergo covalent water expansion (illustrated until reaching the 5 state). These networks can reach water expansion equilibrium while maintaining their original structure. They are retained until an equilibrium state is reached while maintaining the original structure. This provides a remarkable ability to absorb exudate from the wound, promote oxygen flow, and maintain an even moisture level in the injured area. So, you can speed up the healing process and increase the moisture levels in the injured area (Van Vlierberghe *et al.*, 2011). These special physical properties enable the fabrication of hydrogels of various sizes and shapes, facilitating complete coverage of irregularly shaped wounds (Bilici *et al.*, 2016). The hydrogel is biodegradable and biocompatible. It serves as a temporary template during the re-epithelialization and remodeling of chronic wounds. Additionally, the hydrogels exhibit sufficient bioadhesion, which is important to ensure sustained stability. This property improves hemostasis and maintains optimal water content within the wound (Arif *et al.*, 2021). Furthermore, hydrogels provide versatile scaffolds that improve their overall effectiveness in promoting wound healing by incorporating various components such as antibacterial and antimicrobial agents, drugs, and other complementary biomolecules. It can be concluded that hydrogel-based materials are most suitable as bandages to cover skin wounds.

8. Functional Hydrogels for Wound Healing

Traditional wound dressings, including gauze and bandages, are commonly used for dry, clean wounds (Brumberg *et al.*, 2021). Hydrogels, compared with traditional wound dressings, due to their porous structure, have great advantages like biodegradability, drug delivery ability, and sustained release ability (Kamoun *et al.*, 2017). Hydrogels can be used to improve wound healing by tuning their properties and developing hydrogels with characteristics that promote adhesive, anti-inflammatory/antioxidant, antibacterial, hemostatic, angiogenesis, and re-epithelialization. So, they can be tailored to the type of traumatic process (Qian *et al.*, 2020).

8.1. Different Functional Hydrogels for Wound Healing

- Adhesive hydrogels
- Anti-inflammatory/antioxidant hydrogels
- Antibacterial hydrogels
- Hemostatic hydrogels
- Pro-angiogenic hydrogels
- Pro-re-epithelialization hydrogels

8.2. Different Temperature-Responsive Hydrogels

- Photosensitive hydrogels
- Magnetically responsive hydrogels
- pH-responsive hydrogels
- ROS-responsive hydrogels
- Multiple responsiveness hydrogels

8.3. Natural and Synthetic Hydrogels

8.3.1. Natural Hydrogels

- Chitosan
- Gelatin
- Hyaluronic Acid
- Alginate

8.3.2. Synthetic Hydrogels

- Polyethylene Glycol (PEG)
- Polyvinyl Alcohol (PVA)
- Polyvinylpyrrolidone (PVP)

8.3.3. Advanced Hydrogels

- Sprayable Hydrogels
- "Smart" Hydrogels

8.4. Alternative Gels for Wound Healing

- Nanogels
- Aerogels
- Cryogels

8.5. Healing-Promoting Cargoes Carried by Hydrogels

Hydrogels play an important role in promoting wound healing because their porous structure and biocompatibility enable the transport of various biologically active cargoes such as cells and exosomes (Arif *et al.*, 2021; Bilici *et al.*, 2016; Koehler *et al.*, 2018; Van Vlierberghe *et al.*, 2011).

8.6. Some of the Healing-Promoting Cargoes Carried by Hydrogels Are

- Nucleic acids
- Cytokines
- Small-molecule drugs
- Stem cells
- Exosomes
- Nanomaterials

9. Impact of ROS on Skin Regeneration and Wound Healing

Wound healing is a natural and biological process that occurs when the skin tissue is damaged and injured. This process involves blood vessel narrowing and thrombus formation, followed by inflammation, cell proliferation, and ultimately wound reformation. In this process, oxygen is essential in almost every step of the wound healing process. This is because of the energy required for various processes involved in wound healing, including biosynthesis, intracellular transport, and cell movement. ATP synthesis, which serves as the main energy source, is optimized in the presence of oxygen. During wound healing, functions such as cell proliferation and extracellular matrix (ECM) formation increase, and as a result, higher energy requirements emerge. Consequently, the need for oxygen also increases to meet the elevated energy demands during wound healing (Tk, 1969). Oxygen plays an important role in the wound healing process, not only by providing energy but also by supporting many important

enzymatic reactions (Dissemond *et al.*, 2015; Lee *et al.*, 2022).

The potential sources of ROS are mitochondria, endoplasmic reticulum (ER), peroxisomes, various oxidases, and phospholipid metabolism. Additionally, the NADPH oxidase (NOX) family can generate ROS using specific enzymes (Bedard and Krause, 2007). Nitrous oxide enzymes reside on cell membranes and help electrons pass through biofilms. This process leads to the reduction of oxygen and the production of superoxide (O2?). In this pathway, superoxide can then undergo chemical reactions to form various reactive oxygen species, including hydrogen peroxide (H2O2), proxy radical (HO2?), and hydroxyl radical.

These ROS play various roles in cellular functions such as differentiation, proliferation, apoptosis, migration, and contraction (Roy *et al.*, 2006; Soneja *et al.*, 2005). The ROS group includes oxygen derivatives such as hydroxyl radical (OH), peroxide, superoxide anion, and hydrogen peroxide (H2O2). During the non-healing phase of a wound, excessive ROS production can cause cell damage, as ROS molecules oxidize adjacent molecules or cellular components. ROS are widely considered to be the main cause of cell damage during the aging process (Beckman and Ames, 1998). Studies have shown that moderate amounts of reactive oxygen species (ROS) help maintain intracellular balance, but increased ROS levels can negatively impact wound healing processes (Zhu *et al.*, 2019). In other words, as long as ROS levels remain constant, cell integrity and its functions are maintained.

However, ROS also have positive effects. Coordinated production of ROS by immune cells is important and effective for various parts of the body, such as host defense and cell signaling (Jones *et al.*, 2018; Koo *et al.*, 2019). During the homeostatic phase, ROS generated by NADPH oxidase in vascular cells can stimulate the expression of chemotactic and adhesion molecules, thereby reducing local blood flow (Hoffmann and Griffiths, 2018). During the inflammatory phase, neutrophils and macrophages produce superoxide and H2O2, which play important roles in killing bacteria and preventing wound infection. ROS also stimulate the release of tumor necrosis factor alpha (TNF-α) and platelet-derived growth factor (PDGF), supporting cell migration (He *et al.*, 2022; Hoffmann and Griffiths, 2018; Khorsandi *et al.*, 2022; Xu *et al.*, 2020).

During the proliferation phase, ROS/REDOX signaling is required to mediate the tissue growth factor-α1 (TGF-α1) signaling pathway and enhance the expression of fibroblast growth factor (FGF). Furthermore, ROS can stimulate processes such as angiogenesis by expressing vascular endothelial growth factor (VEGF), so endothelial cell division and migration can promote angiogenesis. Therefore, maintaining optimal levels of reactive oxygen species (ROS) is important to combat microorganisms and ensure the survival of cells. Increased ROS at the wound site can lead to the development of chronic inflammation. ROS overactivation impairs the function of transcription factors such as activator protein 1 (AP-1), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and nuclear factor erythroid 2–related factor 2 (Nrf2) (An *et al.*, 2018; Asai *et al.*, 2018). Nrf2 plays an important role in protecting against increased internal oxidative stress and significantly contributes to wound healing (Koike *et al.*, 2020).

On the other hand, stimulation of NF-κB and AP-1 increases the amount of matrix metalloproteinases (MMPs) in dermal fibroblasts, causing degradation of the extracellular matrix (ECM) and slowing the wound healing process. ROS influence inflammatory responses in diabetic models through the NLR family pyrin domain–containing protein 3 (NLRP3), IL-1β pathway (Cheng *et al.*, 2012), and TNF-α (Lord *et al.*, 2017; Seiwerth *et al.*, 2018). Currently, there is a clear desire to restore ROS levels, improve the impaired physiological state around the injury, and promote the wound healing process. According to these data, considering the problems of excessive accumulation of reactive oxygen species (ROS) near wounds, we provide an overview of commonly used materials for ROS removal. These materials are designed to promote cell growth and migration, accelerating the wound healing process by reducing ROS levels within a certain range.

9.1. ROS-Scavenging Materials

- Carbon matrix materials
- Metal nanoparticles
- Small antioxidant molecules
- Antioxidant enzymes
- Natural materials

9.2. Application of ROS-Scavenging Materials in Wound Healing

- Using the main nanomaterials for the purpose of promoting the wound healing process
- Skin wound therapeutics with application of enzymes and peptides with antioxidant properties
- Use of free radical capture treatments for the wound healing process
- Using cells and cell products for the purpose of wound healing

10. Scaffold Properties for Wound-Healing Treatment

Scaffold design plays a central role in wound healing therapy. Generally, scaffolds can be made from syn-

thetic polymers in combination with extracellular matrix components, primarily collagens. These are associated with angiogenesis and revascularization, inflammation, and oxidative stress, and all these functions depend on functionalization with cells, microvascular fragments, or nanoparticles, and conjugation with additives like growth factors, antibacterial, anti-inflammatory, or antioxidant molecules. All of these can be involved in various applications, including antibacterial, antioxidant, and anti-inflammatory properties in microbial infections (La Monica *et al.*, 2024).

10.1. Scaffold Properties for Wound-Healing Treatment

- Promoting angiogenesis and revascularization
- Counteracting the inflammation phase
- Counteracting oxidative stress
- Counteracting microbial infections
- Scaffolding with antibacterial, antioxidant, and anti-inflammatory properties
- Promoting cell proliferation
- Stimulating ECM regeneration
- Particular case (La Monica *et al.*, 2024)

11. Hydrogels as Dressing for Burn Wounds

Hydrogels exhibit three-dimensional, hydrophilic, insoluble structures that are composed of cross-linked polymer chains and swell in aqueous solutions without collapsing (Yao *et al.*, 2021). Hydrogels can be synthesized from natural or synthetic polymers or a combination of both (Chong *et al.*, 2023). Polymer hydrogels have attracted considerable attention in wound dressing research because of their hydrophilicity, ability to mimic the extracellular matrix (ECM), biocompatibility, and biodegradability (Madaghiele *et al.*, 2014; Pan *et al.*, 2021). Hydrogels act as physical barriers, effectively protecting the wound bed from external contamination while creating an ideal moist environment to promote wound healing (Stoica *et al.*, 2020). Additionally, the inherent high-water content of hydrogels provides cooling and soothing effects, making them particularly suitable for burn dressings (Stoica *et al.*, 2020). Extensive references have discussed the polymer sources, cross-linking methods for hydrogel fabrication, and the different types of hydrogels used for wound healing purposes (Firlar *et al.*, 2022; Pan *et al.*, 2021; Yu *et al.*, 2021).

12. Effects of Hydrogels as Transdermal Drug for Burn Wound Healing

Burns that heal are often complicated by infection. Therefore, traditional hydrogel dressings based on the concept of moist wound healing are not sufficient to promote the wound healing process. Burn wound healing in the presence of infection requires administration of drugs and therapeutics such as antimicrobials, growth factors, bioactive compounds, and stem cells, which have shown promising results in promoting burn wound healing (Rowan *et al.*, 2015; Shu *et al.*, 2021; Stoica *et al.*, 2020; Yao *et al.*, 2021). Because burns can affect a variety of body surfaces (Markiewicz-Gospodarek *et al.*, 2022), transdermal drug delivery through the skin-the largest and most easily accessible organ of the human body-is preferred for local or systemic administration and absorption of various drugs and therapeutics in wound healing. Compared to parenteral routes involving needle-based injections, transdermal drug administration is a noninvasive and painless method that supports self-administration without specialist assistance, thereby improving patient compliance (Zaid Alkilani *et al.*, 2015). Furthermore, transdermal administration ensures local and long-acting drug distribution at the wound site through controlled release and permeation of drugs and therapeutics through the skin, while preventing systemic toxic complications and optimizing bioavailability by bypassing pre-systemic metabolism (Wang *et al.*, 2021).

Hydrogels have desirable properties and characteristics such as biocompatibility, biodegradability, non-toxicity, non-allergenicity, ease of application and removal, and high water content, making them suitable as carriers for transdermal drug delivery (Jacob *et al.*, 2021). The moisturizing effect of hydrogels on the skin improves the penetration of therapeutic agents into the skin, thereby facilitating transdermal drug delivery systems (Kim *et al.*, 2020). Furthermore, drugs and therapeutic agents can be incorporated into the porous and cross-linked polymer networks of hydrogels, allowing controlled release by adjusting the porosity, degree of cross-linking, and swelling behavior of the hydrogels (R Johnson and Wang, 2015). External environmental stimuli such as temperature and pH (Zhu and Chen, 2022) can trigger drug release by changing the chemical properties of hydrogels (Firlar *et al.*, 2022). This versatility expands the application of hydrogels as wound dressings (Saghazadeh *et al.*, 2018; Zhong *et al.*, 2020). Drugs and therapeutics incorporated into hydrogels can be classified as: (a) antibacterial agents (b) antioxidants and anti-inflammatory agents (c) analgesics (d) growth factors.

12.1. Hydrogels as Transdermal Drug Delivery Carriers for Burn Wound Healing

- Antimicrobial agents–incorporated hydrogels
- Antioxidant and anti-inflammatory agents–incorporated hydrogels
- Analgesic drugs–incorporated hydrogels
- Growth factors–incorporated hydrogels

13. Burn Wound Healing and Transdermal Drug Delivery

Hydrogels have shown their potential as transdermal drug delivery vehicles for burn wound healing due to their inherent high-water content, which acts as a natural penetration enhancer (Karande and Mitragotri, 2009). However, the unique characteristics of burn wounds pose challenges to the efficacy of transdermal drug delivery. The layer of hard necrotic tissue that forms over the wound bed is a local complication of burn wounds (Saghazadeh *et al.*, 2018). The presence of scar tissue impedes the effective penetration of drugs and therapeutic agents contained in hydrogels into deeper injured tissues (Cartotto, 2017; Souto *et al.*, 2020; Tiwari, 2012). Additionally, high levels of release in burn wounds may further impact the bioactivity and bioavailability of penetrating drugs and therapeutic agents (R Johnson and Wang, 2015; Whittam *et al.*, 2016; Yao *et al.*, 2021).

As a result, efforts are being made to overcome these barriers and improve the transdermal delivery of drugs and therapeutic agents through scar tissue to deeper wound areas, as well as their bioavailability, by using hydrogels as carriers. Various approaches to improvement have been investigated.

13.1. Strategies for Burn Wound Healing

13.1.1. Nanoparticles (NPs)

- Non-polymeric NPs
- Metal and metal oxide NPs
- Lipid-based NPs
- Polymeric NPs

14. Benefits of Nano-Emulsions in Wound Healing

The emergence of innovative therapeutics in the field of wound healing has highlighted the potential of nanotechnology-based drug delivery systems as a viable approach to promote healing mechanisms at various stages. Nano-emulsions are considered an excellent option due to their diverse properties, leading to a wide range of applications in wound treatment.

The main advantages of using nano-emulsions as drug delivery carriers in the treatment and management of wound healing include high drug loading capacity, improved drug solubility and bioavailability, and relative ease of preparation, scaling, and control. They enable controlled drug release and protection from enzymatic degradation. By lowering surface and interfacial tensions and thereby increasing overall viscosity and ductility of the system, nano-emulsions provide thermodynamic stability for the main system. Nano-emulgels offer several advantages over lipid nanoparticles, micro-emulsions, or liposomes for transdermal or dental delivery due to high drug loading capacity, improved diffusivity, permeability, and reduced skin irritation (Gorain *et al.*, 2022; Lo and Fauzi, 2021; McClements and Jafari, 2018). According to references, manufacturing methods of nano-emulsions for wound treatment are broadly divided into two categories: (a) high-energy approaches (b) low-energy approaches.

High-energy methods related to wound healing are further divided into:

- high-pressure homogenization
- ultrasound therapy
- Low-energy methods include:
- phase inversion
- spontaneous emulsification (Qadir *et al.*, 2016; Solans and Solé, 2012)

15. Conventional Methods in Wound Management

Standards of wound care include debridement, irrigation, infection control, and dressings (Margolis *et al.*, 2011). The goal of debridement is to remove nonviable tissue and expose healthy, well-perfused tissue using surgical or autolytic/enzymatic approaches (Stiehl, 2021). After debridement, the wound can be cleaned with saline or sterile water (Maemoto *et al.*, 2023). Detergents, hydrogen peroxide, and concentrated povidone-iodine solutions are not recommended due to tissue damage and their toxicity (Rai *et al.*, 2023). Treating infections is essential for the wound healing process. Failure to control infection results in poor healing and abscess formation. Both topical and systemic antibiotics are used on infected wounds. While topical agents are commonly used for superficial wound infections, systemic antibiotics are used in patients with deep or systemic infections (Powers *et al.*, 2016).

Different types of dressings have been developed to protect wounds from infection and promote the healing process. For example, dry gauze wound dressings have traditionally been used, but removing them can cause secondary damage. Moisture Retention Dressing (MRD) is a material with a water vapor permeability (MVTR) of less than 35 g/m^2/h, allowing wound healing in a moist environment. There are five basic types of MRD, including films, foams, hydrocolloids, alginates, and hydrogels. Some are sticky or absorbent; in other cases, a secondary dressing must be kept in place (Obagi *et al.*, 2019).

Skin substitutes include various biological, synthetic, or biosynthetic materials that can temporarily or permanently cover open skin wounds successfully. These alternatives are valuable in the treatment of both acute and chronic wounds and are useful for covering defects resulting from burns and other injuries, as well as for reconstructive purposes such as resolving extensive post-burn contractures. Split-thickness autografts are often used in chronic wounds. Efficiency is highly dependent on the quality and quantity of donor skin. Donor site pain and infection also limit this approach (Braza and Fahrenkopf, 2019).

The dermal matrix is the underlying tissue that supports the skin. The bioengineered skin matrix is a type of dressing that mimics normal skin structure and promotes wound healing in patients. It is classified into epidermis, dermis, and complete skin. According to the biological source, the substrate can be autologous (the host of the transplant), allogeneic (another human donor), or xenogeneic (another species).

There are several traditional approaches such as plant extracts and herbal medicines that have been used to promote wound healing. Aloe vera is often used in primary care. It has various biological and pharmacological activities, including antioxidant, anti-inflammatory, immunomodulatory, antibacterial, and skin-protective effects, all of which are important in the healing process (Hekmatpou *et al.*, 2019; R. Kumar *et al.*, 2019). One of the herbal formulae developed by the institute is NF3, an innovative herbal formula containing two herbs, astragalus and astragalus, in a 2:1 (w/w) ratio. This formula improved the healing of diabetic ulcers by promoting angiogenesis and inhibiting inflammation through in vitro, in vivo, and clinical studies (Ko *et al.*, 2014; Tam *et al.*, 2015; Tam *et al.*, 2014).

16. Modern Approaches in Wound Healing

In modern treatments, the use of stem cells is becoming a promising candidate. They are characterized by high self-renewal ability and the ability to differentiate into different lineages. In wound healing, the limitations of traditional approaches such as contamination and tissue stimulation can be overcome by promoting tissue regeneration.

16.1. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) were first isolated from bone marrow in the 1970s as mesoderm-derived progenitor cells (Naji *et al.*, 2019). They are able to differentiate into mesenchymal lineages including osteoblasts, chondrocytes, myocytes, and adipocytes, but are unable to differentiate into hematopoietic stem cells. MSCs can be isolated from various tissues such as bone marrow, adipose tissue, peripheral blood, umbilical cord, Wharton's jelly, and dental pulp (P. Kumar *et al.*, 2019).

According to a report published by the International Society for Cell Therapy (ISCT), when cultured in medium containing 10 μL under standard conditions (37 °C, 5% CO2, atmospheric O2 concentration ∼20%) in a humidified incubator (with obtained bovine serum), minimum inclusion criteria to define MSCs include: (a) ability to adhere to plastic surfaces under standard culture conditions (b) presence of surface markers such as CD73, CD105, CD90, and absence of markers such as CD14, CD19, CD34, CD45 (c) ability to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro under specific medium conditions (Dominici *et al.*, 2006) MSCs exhibit therapeutic effects through immunomodulatory, anti-inflammatory, pro-angiogenic, antioxidant, and anti-apoptotic activities (Mishra *et al.*, 2020). These effects are expressed through paracrine activity rather than direct cell differentiation. The secretome or conditioned medium (CM) is a complex product secreted by MSCs. It consists of soluble proteins (mainly growth factors, cytokines, and chemokines) and extracellular vesicles (EVs) in which proteins, lipids, and genetic material are encapsulated and transported. Although many skin substitutes are available on the market, stem cells are considered a better option for wound healing. They have been shown to promote healing of various types of wounds, including acute wounds, chronic wounds, and burns. Living cells are used that can actively interact with the wound environment and promote healing through the secretion of growth factors and other signaling molecules. This approach has proven effective in treating chronic wounds that do not respond to conventional treatments.

In addition, cell-free products such as growth factors, cytokines, and extracellular vesicles can be used to stimulate the body's natural healing mechanisms and functions without the need for living cells (Mazini *et al.*, 2020). In contrast, many commercially available skin substitutes are often made from synthetic materials that lack the biological complexity and versatility of living cells.

16.2. Advantages of MSC-Based Cell-Free Therapy

Although using cell-based products to promote wound healing is not new, there are still hurdles to overcome. Most commercially available cell-based skin graft substitutes such as Apligraf®, Recell®, PolyActive®, and OrCel® are expensive (Oualla-Bachiri *et al.*, 2020). Storage and transportation require special and strict conditions. In addition, potential risks such as tumorigenicity, rejection, and infection complicate frequent use. Delivering living cells to the wound site is also a challenge. Injecting cells through a needle damages cell membranes and viability (Wahlberg *et al.*, 2018). After injection, excess apoptotic or necrotic cells can trigger a local immune response and cause secondary damage. Therefore, different cell-free approaches are required. Numerous preclinical studies have demonstrated the therapeutic efficacy of MSC-derived cell-free products (MSC-CM). Various animal models have been used to study their effectiveness and potential. Compared to cell-based products, MSC-CMs can be more easily manufactured, packaged, stored, and transported. This allows for large-scale production. Cell-free products are treated as drugs, making quality control easier. Donor and recipient matching is not required, and immune rejection can be avoided. Furthermore, they can reduce the possibility of embolization, tumorigenicity, and infection transmission (Abbasi-Malati *et al.*, 2018). Therefore, unlike cell-based products, MSC-CM can be considered a ready-to-use pharmacological agent. Generally, isolated cell-free products can be stored for 6 to 7 months at temperatures between -20 °C and -80 °C (Torizal *et al.*, 2022).

16.3. Continuous Oxygen Therapy

In wounds, continuous delivery of 98–100% oxygen to the wound bed at a rate of 3–15 ml/h requires patients to wear a small wearable device attached to the wound 24 hours a day for a predetermined number of days. Of the 49 included studies, continuous oxygen therapy was examined to treat different wound types, with the most common being diabetic foot ulcers (DFU) (n=10), followed by venous leg ulcers (n=10), surgical wounds (n=1), and other wounds (n=1). Four studies used Natrox, four used TransCu O2, two used unspecified transdermal continuous oxygen therapy, one used Epiflo, one used micro-oxygenation devices, and in one case, oxygen-perfused negative pressure was used. Nine of the 13 studies were conducted as RCTs, while two RCTs (Niederauer *et al.*, 2015, 2017) were interim results of a larger RCT using TransCu O2 in DFU (Niederauer *et al.*, 2018). This review presents only the final RCT results to prevent double counting. After 12 weeks, improved wound healing was observed in DFUs. A significantly higher percentage of DFUs healed in the treatment group compared to placebo (32.4% vs. 16.7%, p=0.033). The time to 50 μU was significantly reduced in patients receiving CDO therapy (mean 18.4 days vs. 28.9 days, p=0.001) (Niederauer *et al.*, 2018). Yu *et al.* (2016) conducted an RCT in 20 patients and showed that the use of Natrox resulted in a 100% healing rate of grade II diabetic foot ulcers in the treatment group compared to 0% in the control group. A randomized clinical trial by Driver *et al.* (2013) using Epiflo in 17 patients with diabetic foot ulcers concluded a statistically significant difference in mean wound size reduction of 87% (55.7%–100%) in the treatment group compared to 46% (15%–99%) in the control group (p<0.05) (Driver *et al.*, 2017). Driver also conducted an RCT evaluating transcutaneous continuous oxygen therapy in 130 DFU patients. After 12 weeks, 54% of wounds in the treatment group were completely healed compared to 49% in the control group, although the results were not statistically significant (p=0.4167). A randomized controlled trial of 145 DFU patients using Natrox (Serena *et al.*, 2021) reported complete wound closure at 12 weeks in 44.4% of the TOT group compared to 28.1% in the standard treatment group (p=0.044). In a recent RCT by Al-Jalodi *et al.* (2022), 28.1% of patients in the SOC group healed in 12 weeks compared to 44.4% in the SOC plus TOT group (p=0.044). Furthermore, 85% of patients in the TOT group remained healed after 1 year compared to 60% in the control group. He and 24 colleagues conducted a clinical trial using a micro-oxygenation device in DFU. The mean wound healing time was significantly reduced when standard care was combined with continuous oxygen therapy (p<0.05), with a 10% reduction in scar length after 4 weeks (88.8% in the intervention group vs. 28.5% in the control group, p=0.049). In an RCT by Wang and Yu, the use of negative pressure showed statistically significant results in reducing wound site area and depth (all p<0.01), and granulation tissue increased in more hospitalized patients (p<0.01). A prospective comparative study in 27 cases showed that transcutaneous continuous oxygen therapy for venous leg ulcers promoted wound closure (p<0.001).

16.4. Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy was used in seven studies and administered at high pressures of 5 to 50 mbar. The most commonly used device was TWO2 (n=5), which delivered oxygen via a chamber. Two other studies used unspecified topical pressurized oxygen therapy. In an RCT by Frykberg *et al.*, 29 of 73 patients with DFU were treated using the TWO2 HyperBox. The closure rate at 12 weeks was 41.7% in the treatment group compared to 13.5% in the sham group (p=0.010). In an RCT by Azimian *et al.*, unspecified hyperbaric transcutaneous oxygen therapy was applied using humidified oxygen at high pressure (10 L/min) for 20

minutes, three times over 12 days. Treatment of 100 grade II–IV pressure ulcers resulted in complete closure in 16/50 ulcers in the treatment group compared to 1/50 in the control group ($p<0.001$). A prospective controlled study of DFU in 31 patients showed a statistically significant difference in complete epithelialization between treatment and control groups (82.4% vs. 45.5%, $p=0.04$). Two comparative TWO2 studies on venous leg ulcers demonstrated a statistically significant reduction in MRSA infection rates. In one study, 80% of ulcers healed completely in 12 weeks in the TWO2 group compared to 35% in the compression bandage group ($p<0.0001$). Pressure Wound Therapy (NPWT) using conventional NPWT and native NPWT with additional local barometric oxygen therapy showed that patients receiving native NPWT with topical oxygen achieved the highest average reduction in wound area and depth and formed more sterile cultures of infected wounds. A matched group study by Yellin *et al.* concluded that the control group (no TWO2) had a 9 times higher risk of hospitalization and 5 times higher risk of amputation than the TWO2 group.

16.5. Oxygen Dressings

Two studies used oxygen dressings: Oxyzyme and OxyBand. Oxyzyme is an oxygen-enriched hydrogel dressing containing glucose oxidase to generate hydrogen peroxide. Upon contact with the wound surface, hydrogen peroxide is converted to water and dissolved oxygen by serum catalase. OxyBand is a multi-layered dressing pre-loaded with pure oxygen that is continuously delivered to the wound bed. It acts as a reservoir that releases oxygen. Although the Oxyzyme/Iodozyme RCT by Moffatt *et al.* in 40 cases showed no statistically significant difference in 12-week cure rates compared to standard treatment (49.1% control vs. 44.7% treated), the frequency of dressing changes and average cost per patient were reduced in the treatment group. A prospective, randomized, controlled, open-label, single-center study by Lairet *et al.* in 17 patients with foot ulcers showed that OxyBand reduced the mean time to complete healing (9.3 ± 1.7 days vs. 12.4 ± 2.7 days in the Xeroform group, $p<0.001$). Pain scores on postoperative day 4 and day 12 were also significantly lower (both $p<0.05$).

16.6. Oxygen Transfer

Oxygen transfer refers to the delivery of oxygen gas to the wound surface by spray. Five studies investigated the effectiveness of hemoglobin sprays. Four evaluated Granulox (Infirst Healthcare), and one evaluated Granulox plus a 10% aqueous solution containing carbonylated hemoglobin. Each study examined hemoglobin spray on different wound types: foot ulcers, venous leg ulcers, scabies wounds, diabetic foot ulcers, and chronic wounds. Jonker *et al.*'s RCT in 42 cases investigated twice-weekly use of Granulox for foot ulcers but found no significant improvement after 12 weeks. The control group achieved a higher cure rate (8/14 vs. 4/15, $p=0.14$). The mean reduction in ulcer size was 100% in the control group compared to 48% in the treatment group ($p=0.21$). A retrospective cohort study of 45 cases showed that Granulox improved cure rates at 26 weeks (90% treated vs. 38% control, $p<0.001$). The median time to complete wound healing was 11.4 weeks in the treatment group compared to 6.6 weeks in the control group.

17. Wound Healing Techniques

17.1. High Energy Methods

High-energy approaches use mechanical force to provide large destructive energies. Droplet size depends on the equipment, preparation parameters such as temperature and time, and sample properties. High-energy methods are complex and energy-intensive. This makes them expensive and unsuitable for heat-sensitive products (Jasmina *et al.*, 2017). These methods fall into the categories of high-pressure homogenization and ultrasound. This technique is widely used to develop nanoemulsions using different forces, namely pressure. The system can generate particle sizes of approximately 1 nm, as well as intense turbulence, cavitation, and hydraulic shear. A high-pressure piston/homogenizer is used to force two liquids consisting of a surfactant and a co-surfactant into a small orifice at intense pressure (500–5000 psi), forming an interface between water and oil and transforming it. The extent to which the formed droplets are broken into smaller droplets depends on the force applied. The development of nanoemulsions by high-pressure homogenization methods is not impulsive in nature, as it requires force input in the form of chemical or mechanical energy. Furthermore, the preparation may become unstable if the system temperature increases during processing (Jaiswal *et al.*, 2015; Jasmina *et al.*, 2017). This technique has wide applications in wound treatment using herbal active ingredients such as betulin-enriched extract and oil palm leaf (Elaeis guineensis Jacq). Emulsification using this technique significantly reduces droplet size and can impact targeted drug delivery. Optimal drug loading and polydispersity index were also observed (Vater *et al.*, 2022; Zain *et al.*, 2021). Ultrasound nanoemulsions can be easily produced using ultrasound technology. Sound waves are used to move molecules and later break up oil droplets through local turbulence. Typically, a probe, metal horn, generator (which generates radio waves), and piezoelectric transducer are used to generate nanoemulsions via ultrasound (Zain *et al.*, 2021). In con-

trast to high-pressure homogenization methods, ultrasound requires less energy, is easy to use, and is cost-effective. Energy is delivered using ultrasound probes called sonotrodes. These contain a piezoelectric crystal that expands and contracts in response to AC energy. When the tip of an ultrasonic device contacts a liquid, mechanical vibrations and cavitation occur. Despite its power, this approach is still limited to laboratory research and pilot-scale production due to wave effects around molecules that can affect large-scale manufacturing (Che Marzuki *et al.*, 2019; Gharibzahedi and Jafari, 2018; Jaiswal *et al.*, 2015). Using this method, various synthetic nanoemulsions (e.g., clindamycin) and herbal ingredients (e.g., eugenol) were prepared and found effective for wound healing with optimal viscosity and high skin compatibility. Transmission electron microscopy analysis also showed that the formed nanoemulsion was spherical and had reduced droplet size (Abdellatif *et al.*, 2021; Ahmad *et al.*, 2018).

17.2. Low Energy Methods

Low-energy techniques exploit the properties of surfactant, oil, and water systems and do not require special equipment. Due to their low cost and ease of implementation, these techniques have led to increased research into nanoemulsions for development and broad applications. Low-energy emulsification techniques are energy-efficient as they exploit the chemical energy inherent in the system and produce nanoemulsions with only gentle stirring. These methods can be categorized into phase inversion and natural emulsification techniques. Phase inversion and phase penetration techniques create nanoemulsions on the surface by changing the spontaneous curvature of the surfactant. In this technology, the system temperature is changed using non-ionic surfactants. At high temperatures, oil-in-water (O/W) emulsions transform into water-in-oil (W/O) emulsions. Phase inversion emulsification requires a critical surfactant concentration. Increasing temperature can dehydrate the polymer chains of the surfactant and increase its lipophilicity. At low temperatures, a large positive spontaneous curvature is formed. A sufficient phase transition can be generated either by changing the composition at a constant temperature or by changing the temperature at a stable composition. Additionally, in some cases, generating droplet sizes in the micron range using phase inversion requires high energy input, which can be achieved using high shear agitation or an ultrasonic generator. This method is widely used in wound dressings to deliver active ingredients such as phenytoin and chlorhexidine. Formulations prepared using this technique were found to be stable and highly monodisperse. Enhanced drug entrapment efficiency was observed with no evidence of precipitation. Nanoemulsions produced using this method showed improved wound healing (Teo *et al.*, 2017). Natural emulsification, also called self-emulsification, requires no external energy input. Droplet formation occurs by contacting two immiscible liquids that are not in equilibrium. One of the greatest advantages of this technique is that nanoemulsions can be prepared without special equipment and developed effectively at room temperature. However, the presence of solvent and low formation of the oil phase are two major drawbacks of the self-emulsification technique. This method has been widely used to develop nanoemulsions formulated with herbal active ingredients for wound healing. After sonication for 30 minutes, a stable and transparent nanoemulsion was obtained. Droplet size analysis showed a large surface area, which improved absorption through skin texture. Quantitative analysis of nanoemulsions in the UV and visible wavelength range showed that absorption decreased with increasing sonication time (Bonferoni *et al.*, 2018; Shanmugapriya *et al.*, 2018; Sugumar *et al.*, 2014).

17.3. Advanced Therapies for Wound Healing in Preclinical and Clinical Studies

17.3.1. Advanced Wound Therapies in Preclinical Trials

In conditions such as acute wounds (e.g., surgical and traumatic wounds), dressings control bleeding, absorb exudate, and effectively close wounds to promote the healing process. Recent advances in wound dressings for acute wounds have focused on hemostasis, absorption of wound exudate, and firm wound closure for infection control. For example, a highly adhesive wound dressing composed of alginate and poly(N-isopropylacrylamide) contracted the wound in mouse splint models due to its thermoresponsiveness and high toughness, accelerating wound contraction. Recent attempts to combine adhesive hydrogels with surgical mesh-such as poly(N-isopropylacrylamide)/chitosan hydrogel and polyethylene terephthalate surgical mesh-demonstrated strong adhesion, flexibility, permeability, and strength. These are targeted for chronic wounds, where advanced dressings aim to address the disorganized inflammatory stage, replace skin tissue, and protect against infection. For diabetic wounds, recent focus has been on stimulating the healing process by inducing acute inflammation. Preemptive administration of a mast cell stabilizer and release of the neuropeptide substance P both resulted in severe post-injury inflammation, improved wound re-epithelialization, and accelerated wound healing in diabetic mice (McClements and Jafari, 2018; Whittam et al., 2016). Additionally, removing tissue-damaging pro-inflammatory factors improved tissue regeneration and healing in diabetic mice. In several diabetic mouse models, reactive oxygen species and de-

creased matrix metalloproteinase 9 (MMP9) activity-both continuously released by immune cells—promoted progression to the proliferative phase and enhanced wound healing. Sustained-release hydrogels of deferoxamine, an iron (II) scavenger that inhibits the conversion of hydrogen peroxide to highly toxic hydroxyl radicals, and hydrogels that release small molecules such as MMP9 inhibitors and MMP9-silencing RNA, improved re-epithelialization and promoted wound healing in diabetic mice (Gorain et al., 2022; Lo and Fauzi, 2021; Solans and Solé, 2012). A sustained-release formulation of PPCN hydrogel with stromal cell-derived factor-1 also promoted wound healing in diabetic mice. Dressings that remove pro-inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 through electrostatic interactions demonstrated improved wound closure and reduced chronic inflammation in db/db mice. Hydrogels decorated with the heparin-binding domain of laminin, with or without encapsulated VEGF and PDGF, showed enhanced wound healing after topical application in db/db mice. Thermo-responsive hydrogels modified with conjugated laminin-derived peptide A5G81 promoted migration of keratinocytes and dermal fibroblasts, enhancing wound healing in splinted wounds in db/db mice. A peptide derivative of heat shock protein 90α applied to porcine burn wounds reduced cell apoptosis and inflammation using topical carboxymethyl cellulose hydrogels, resulting in re-epithelialization in this large animal model. Infections with potentially fatal consequences often occur in both acute and chronic wounds. Various anti-infective dressings have shown promising results in preclinical studies. Polymer hydrogels composed of poly (acrylic acid) and poly(acrylamide) loaded with antimicrobial silver/graphene particles exhibited high swelling rates and promoted wound healing in excised rat wounds. New hemostatic, absorbent, antimicrobial wound dressings based on novel mechanobiological strategies have shown promise in animal models of surgical wound healing. Two recently reported agarose and alginate hydrogel systems demonstrated high drug loading and sustained release of three antibiotics, along with good wound closure and positive effects on burn injuries in a pig model (Braza and Fahrenkopf, 2019; Obagi et al., 2019). A suspension of multilayered poly-L-lactic acid nanosheets has been reported to have high barrier function, adhering firmly to burn wounds without adhesive and suppressing Pseudomonas aeruginosa infection in mice for at least 80 days. For wound infections, several therapeutic conductive dressings have been developed to sense infection-related parameters such as pH and temperature and release antibiotics when necessary (R. Kumar et al., 2019; Tam et al., 2015; Tam et al., 2014). One hydrogel system, based on a carbon/poly-aniline working electrode, could measure wound pH and release cefazolin, promoting wound healing in an excision mouse model. Another system used electrical stimulation to provide predictive cues and improve wound healing in diabetic mice. Recently, various wound dressings have been developed that integrate electronics for electrical stimulation, wound monitoring (e.g., pH and temperature), and on-demand drug delivery (Dominici et al., 2006; P. Kumar et al., 2019; Naji et al., 2019). Additionally, several antimicrobial peptides have shown promise in preclinical wound models (Mazini et al., 2020; Mishra et al., 2020; Oualla-Bachiri et al., 2020). Antimicrobial peptide-releasing DNA hydrogels, whose retention mechanism relies on ionic interactions between negatively charged DNA and cationic antimicrobial peptides, reduced S. aureus burden in ex vivo porcine skin explants and promoted wound healing in mice.

17.3.2. Advanced Wound Therapies in the Clinical Pipeline

Several advanced wound treatments are currently in clinical trials. A search of clinical trial databases was conducted to identify the most frequently studied interventions targeting wound management, anti-infectives, and biologics. This section summarizes ongoing clinical trials across various wound types. Advanced anti-scarring and pro-healing therapies for surgical wounds are in development. OLX101 is a cell-permeable asymmetric interfering RNA that targets connective tissue growth factor (CTGF) to counteract hypertrophic scarring (OliX Therapeutics). Unlike liposomal or nanoparticle delivery systems, OLX101 is a small interfering RNA that enters cells spontaneously without complex delivery mechanisms. It is currently being developed as an intradermal injectable for hypertrophic and keloid scars. New peptide formulations are also under investigation to promote wound closure and reduce scarring. One example is SLI-F06 (Scarless Laboratories), a fibro-modulin (FMOD)-based amino acid peptide sequence that stimulates fibroblast and endothelial cell migration, myofibroblast differentiation, and contraction. In preclinical studies, intradermal administration of FMOD in a pig wound model reduced scar size, increased tensile strength, and improved dermal collagen organization.

18. Additional Peptides Currently in Development

Additional peptides in development include a Connexin43 (Cx43) mimetic peptide (Granexin). Cx43 is commonly found in the epidermis and dermis, and chronic wound studies have shown its presence at wound edges. Both Cx43 and peptidomimetics of its carboxyl terminus have improved wound closure rates and reduced scar formation. Granexin is being studied in venous leg ulcers, diabetic foot ulcers, and surgical

wounds in phase 1 and phase 2 trials. For soft tissue defects and refractory wounds, several stem cell, exosome, and peptide therapies are in clinical trials. ADSC-SVF-002 (AdiSave) is an autologous fat-derived stem cell therapy injected subcutaneously into soft tissue defects and abnormally healing wounds, with or without unprocessed autologous fat. A single-arm, open-label, single-center safety study is ongoing to evaluate its safety in subjects with soft tissue defects. Unlike cell-based therapies, platelet-derived exosomes are being tested for advanced wound healing (Plexoval, ExoPharm Limited). Autologous exosomes are delivered by local injection over six weeks to study safety, wound closure, and scarring. Advances in surgical wound healing without biologics are also being investigated. Portable NPWT devices such as PICO (Smith and Nephew) achieved non-inferiority in a phase 4 multicenter randomized controlled trial, showing superior wound healing progression over a one-week treatment period. An ongoing RCT is evaluating infection incidence over 1–3 months for surgical site infection prevention after cardiac surgery using extracorporeal circulation, compared with disposable hydrocolloid dressings. Hyperthermia or non-contact normothermia (38°C dressings) have shown promising results in preliminary studies and clinical trials for pressure ulcers and venous stasis ulcers (Driver et al., 2013; Yu et al., 2016). It was hypothesized that warming agents applied to semipermeable dressings (Active Wound Therapy) would increase blood flow and immunogenicity, enhancing healing (Driver et al., 2017; Serena et al., 2021). However, recent studies using similar technologies such as infrared therapy have shown mixed results (Al-Jalodi et al., 2022; Driver et al., 2013), and modern clinical implementation remains limited.

Several diabetic foot ulcer treatments are under investigation. An FDA-approved eye gel containing the beta-adrenergic antagonist timolol is being tested in a phase 3 trial, as beta-blockers have shown angiogenesis and tissue repair benefits in vitro and in vivo. Topical bacteriophage dispersion is in phase 1/2a trials, aiming to reduce infections by targeting Pseudomonas aeruginosa, Staphylococcus aureus, and Acinetobacter baumannii. A topical dispersion of genetically engineered Lactococcus lactis bacteria (AuP1602-C) is also in phase 1/2a trials. These bacteria express three proteins: fibroblast growth factor 2, interleukin 4, and macrophage colony-stimulating factor 1. As an alternative to surgical debridement, a protease-containing wound solution is being investigated for outpatient use in a phase 2 study. In an RCT, topical application of ON101 cream-containing two plant extracts that polarize macrophages to the M2 phenotype-was associated with absorbable wound dressing and showed improved healing after 16 weeks. Various biologic dressings based on autografts, allografts, phages, bacteria, and drug-releasing systems for diabetic foot ulcers are under clinical investigation. Next-generation debridement therapy for burns is in clinical trials. NexoBrid (KMW-1) is a topical drug composed of proteolytic enzymes isolated from pineapple stem (Bromelain). These proteins include at least four cysteine proteinases that hydrolyze and solubilize heat-denatured proteins in scabs (Vater et al., 2022; Zain et al., 2021). NexoBrid allows selective and rapid removal of dead or damaged tissue within 4 hours. In a phase 3 trial, 89% of patients had complete scab removal without serious adverse events. Several cell-based therapies are under investigation to improve burn wound healing. StrataGraft (Mallinckrodt) is a bi-layer cellularized scaffold containing keratinocytes and dermal fibroblasts, applied topically to recruit endogenous skin cells. Other technologies include Epicel (Genzyme Corp) and Engineering Skin Substitute (ESS) (Amarantus Bioscience Holdings), which are tissue-engineered skin constructs made from the patient's epithelial cells and collagen-containing fibroblasts. In preclinical studies, ESS created a functional skin barrier. A completed clinical trial evaluated its use in treating severe burns (up to 95% body surface area) in pediatric patients. A phase 2 study is underway to compare ESS with autografted mesh gap skin for treating life-threatening burns. Finally, SkinMed (BioDan) is based on autologous fibroblasts and keratinocytes obtained from a single biopsy and implanted into clotted human plasma as a 3D skin scaffold.

19. Conclusion

The main research focus is on skin wound substitutes to replace invasive autografts. This approach offers a new option for severe burns or other deep injuries where autografts and allografts are currently the standard of care treatments. Recently, three-dimensional (3D) bioprinting has received significant attention in this field by combining scanning and printing approaches to create a personalized system that allows complete coverage of wounds in three dimensions for skin replacement. A portable 3D scanning and bioprinting system is capable of printing autologous fibroblasts (dermis) and keratinocyte cell layers (epidermis) from collagen and thrombin cross-linked fibrinogen. It is designed for successful vascularization and re-epithelialization and demonstrated improvement in mouse wound models (Albanna *et al.*, 2019). Bioprinted gelatin–alginate hydrogel containing mesenchymal stem cells and serving as an angiogenic nitric oxide source promoted re-epithelialization and wound closure in murine burn wounds (Wu *et al.*, 2021). The large mesh size of the gel used as bio-ink can cause burst release of the drug, so the hydrogel was cross-linked during the printing process to sustain drug release. A 3D-printed photo-crosslinked hydrogel composed of

chitosan–methacrylate, the antibiotic levofloxacin, and the analgesic lidocaine demonstrated sustained drug release and accelerated wound closure over 3 days in rat burn wounds (Teoh *et al.*, 2022).

Overall, experimental dressings for acute and chronic wounds with immunomodulatory, anti-infective, skin replacement, and sealing properties have shown promise in animal models of wound healing. These proof-of-concept studies illustrate the growth of the preclinical pipeline and highlight the potential for studying key properties in the pathophysiology and clinical pathology of acute and chronic wounds. According to all these data, advanced modern activities for wound healing are important because they can accelerate the healing process and prevent wound progression. This article can serve as a useful reference for recent studies on advanced modern treatments for wound healing in various conditions.

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REVIEW ARTICLE

Apple Pomace-Derived Fiber in Ruminant Nutrition: Processing, Biological Mechanisms, and Economic Implications

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Abstract

Apple pomace, a major by-product of fruit processing, has emerged as a promising source of dietary fiber for ruminant nutrition. This review explores its physicochemical characteristics, processing techniques for feed integration, and biological effects on rumen fermentation, nutrient digestibility, and animal performance. Comparative insights with conventional fiber sources highlight apple pomace's fermentability, antioxidant potential, and prebiotic properties. Furthermore, its utilization supports circular agriculture by reducing feed costs and valorizing agro-industrial waste. In addition to its high pectin content and moderate antioxidant activity, apple pomace provides fermentable carbohydrates that stimulate volatile fatty acid production, enhance microbial protein synthesis, and support immune modulation. Economic analyses indicate that partial replacement of conventional feed ingredients with dried apple pomace can lower ration costs by 5–12% in apple-producing regions. Despite its potential, challenges remain in standardizing composition, ensuring palatability, and optimizing inclusion rates. Future research should focus on long-term health outcomes, regional formulation strategies, and scalable processing technologies to fully realize its role in sustainable livestock production.

1. Introduction

The global livestock sector is increasingly challenged by feed resource limitations, environmental pressures, and the need for sustainable intensification. Conventional feed ingredients such as cereal grains and soybean meal are associated with high production costs, land-use competition, and ecological concerns (IFIF 2020). In response, agro-industrial by-products have gained attention as alternative feed resources that align with circular economy principles and sustainable agriculture (Vendruscolo *et al.*, 2008).

Apple pomace, the solid residue remaining after juice and cider extraction, represents a significant agro-industrial by-product with high nutritional potential. Rich in dietary fiber, pectin, polyphenols, and fermentable carbohydrates, it has attracted increasing attention as a sustainable feed ingredient for ruminants (Szymańska-Czerwińska *et al.*, 2024; Vendruscolo *et al.*, 2008). The valorization of apple pomace aligns with circular agriculture principles, offering both environmental and economic benefits by reducing feed costs and minimizing waste (Szymańska-Czerwińska *et al.*, 2024).

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Recent studies have demonstrated that apple pomace can positively influence rumen fermentation, microbial activity, and nutrient digestibility when properly processed and incorporated into ruminant diets (Tiwari, et al. 2008). Its antioxidant and prebiotic properties further enhance its functional value, contributing to improved animal health and productivity (Szymańska-Czerwińska *et al.*, 2024). However, variability in chemical composition, palatability concerns, and limitations in large-scale processing remain key challenges to its widespread adoption (Vendruscolo *et al.*, 2008).

This review aims to synthesize current knowledge on the physicochemical characteristics of apple pomace, evaluate processing techniques that enhance its feed value, and assess its biological effects on dairy and beef cattle. Additionally, the economic implications and future research directions for its integration into sustainable feeding systems are discussed.

2. Composition and Functional Properties of Apple Pomace Fiber

Apple pomace contains a complex matrix of dietary fiber and bioactive compounds that contribute to its nutritional and functional value in ruminant diets. The major fiber components include cellulose, hemicellulose, pectin, lignin, and polyphenols, each playing distinct roles in rumen physiology and microbial dynamics (Fidriyanto *et al.*, 2023; Murad and Azzaz, 2011).

Cellulose and hemicellulose are structural polysaccharides that stimulate chewing activity and saliva production, thereby enhancing rumen motility and buffering rumen pH (Fidriyanto *et al.*, 2023). Pectin, a soluble fiber, is rapidly fermented in the rumen, increasing the production of volatile fatty acids, particularly propionate, which serves as a key precursor for glucose. Lignin, although indigestible, contributes to rumen fill and satiety but can reduce overall diet digestibility when present at high levels. Polyphenols, found in moderate concentrations, possess antioxidant and anti-inflammatory properties that may help reduce oxidative stress and support immune function (Fidriyanto *et al.*, 2023; Murad and Azzaz, 2011; Schmid *et al.*, 2020; Szymańska-Czerwińska et al., 2024).The typical composition of apple pomace (on a dry matter basis) includes crude fiber (15–25%), pectin (6–10%), crude protein (5–8%), total sugars (10–15%), and polyphenols (0.5–1.5%). These components and their functional roles are summarized in Table 1 (Fidriyanto *et al.*, 2023; Murad and Azzaz, 2011; Szymańska-Czerwińska *et al.*, 2024). While the protein content is relatively low and requires supplementation, the fermentable sugars and fiber fractions provide readily available energy and functional support for rumen microbes (Gadulrab *et al.*, 2023).

From a functional perspective, apple pomace fermentation yields VFAs such as acetate (linked to milk fat synthesis), propionate (gluconeogenesis), and butyrate (rumen epithelium health) (Gadulrab *et al.*, 2023). Pectin also promotes the growth of beneficial microbial populations including Lactobacillus and Bifidobacterium, contributing to improved gut health and immune modulation (Fidriyanto *et al.*, 2023). Additionally, polyphenols reduce oxidative stress, potentially improving reproductive performance and meat shelf life (Fidriyanto *et al.*, 2023; Kruczek *et al.*, 2017).

Table 1
Chemical composition and functional roles of apple pomace fiber components (DM basis)

Component	Range (% DM)	Functional role
Crude fiber	15-25	Stimulates rumen motility
Pectin	6-10	Enhances microbial fermentat
Crude protein	5-8	Requires supplementation
Total sugars	10-15	Provides fermentable energy
Polyphenols	0.5-1.5	Antioxidant and anti-inflammatory effects

3. Processing Techniques for Feed Application

Apple pomace requires appropriate processing to ensure its stability, digestibility, and suitability for ruminant feed. Drying reduces the moisture content of fresh apple pomace (typically 70–80%) to below 10%, significantly improving shelf life, microbial stability, and transportability. Techniques include solar drying, which is cost-effective but weather-dependent; hot-air drying, offering controlled and scalable conditions; and drum drying, suitable for industrial-scale operations with consistent output and low energy loss. Drying also affects grinding efficiency and powder morphology, which are critical for feed uniformity and digestibility (Shalini and Gupta, 2010; Tulej and Głowacki, 2022) .

Mechanical processing such as grinding and pelletisation improves particle size uniformity, feed texture, and overall palatability in mixed rations. Grinding reduces particle size and enhances mixing efficiency, while pelletisation increases bulk density, improves intake, and reduces feed sorting. Biological fermentation with lactic acid bacteria or yeast improves digestibility, reduces anti-nutritional factors such as tannins, and enhances probiotic effects. Mixed silage containing dried apple pomace and brewers' spent grains has been shown to improve lactic acid production, protein digestibility, and metabolizable energy in ruminants (Dai *et al.*, 2022).

Table 2 compares the nutritional and functional attributes of apple pomace with conventional fiber

sources such as wheat bran, beet pulp, and alfalfa hay. It highlights apple pomace's higher pectin content, moderate antioxidant activity, and strong prebiotic potential, which distinguish it from other fiber sources.

References: Kalia & Gupta (2005) and Szymańska-Czerwińska *et al.* (2024).

Apple pomace stands out for its high pectin content, moderate antioxidant activity, and strong prebiotic potential, making it a valuable addition to ruminant diets when processed appropriately (Fidriyanto, *et al.* 2023).

4. Biological Mechanisms and Performance Effects of Apple Pomace in Ruminant Nutrition

pple pomace fibre stimulates microbial fermentation in the rumen, increasing the production of acetate and butyrate, which are key volatile fatty acids (VFAs) involved in milk fat synthesis and epithelial health. Pectin, a rapidly fermentable fibre, promotes the growth of cellulolytic bacteria such as Fibrobacter succinogenes and Ruminococcus albus, thereby enhancing fibre degradation and energy yield (Fidriyanto *et al.*, 2023; Gadulrab *et al.*, 2023). The high fibre content of apple pomace also increases chewing time, which in turn enhances saliva flow and buffers rumen pH. This buffering action is critical for preventing subacute ruminal acidosis (SARA), particularly in high-concentrate diets (Zebeli *et al.*, 2012).

Fermentable carbohydrates in apple pomace support microbial growth, contributing to microbial protein synthesis and improved nitrogen utilisation. This mechanism is especially beneficial in diets with low crude protein, where microbial synthesis can compensate for protein deficits (Dai, et al. 2022). In addition, polyphenols such as quercetin and chlorogenic acid present in apple pomace may reduce oxidative stress and inflammation, particularly in high-producing dairy cows under metabolic strain. These compounds enhance immune function and may improve reproductive efficiency (Boyer and Liu, 2004; Fidriyanto *et al.*, 2023). Table 3 summarises the main biological mechanisms and contributions of apple pomace in ruminant nutrition.

5. Effects on Dairy and Beef Cattle

In dairy cattle, inclusion of dried apple pomace at levels up to 15% of dry matter (DM). in total mixed rations (TMR) has been shown to maintain or slightly improve milk yield and fat content in lactating cows. This effect is attributed to increased acetate production from fibre fermentation, which supports milk fat synthesis, and propionate production from pectin, which enhances glucose availability for lactose synthesis (Gadulrab, et al. 2023). Rumen health is positively influenced by the high fibre and low starch content of apple pomace, which promotes chewing activity and saliva flow, thereby stabilising rumen pH and reducing the risk of subacute ruminal acidosis (SARA) (Zebeli *et al.*, 2012; Allen, 1997).

Table 2
Comparative analysis of apple pomace and conventional fiber sources (DM basis)

Parameter	Apple pomace	Wheat bran	Beet pulp	Alfalfa hay
Dry Matter (%)	∼20 (fresh) / >90 (dried)	∼88	∼90	∼85
Crude fiber (% DM)	15–25	10–12	16–20	25–30
Pectin content (% DM)	6–10	<2	15–20	<1
Lignin (% DM)	5–8	3–5	2–4	8–10
Fermentability	Moderate–high	Moderate	High	Moderate
Energy density	Low–moderate	Moderate	High	Moderate
Protein (% DM)	5–8	14–16	8–10	17–20
Antioxidant content	Moderate	Low	Low	Low
Cost (per ton)	Low (by-product)	Moderate	Moderate–high	High
Prebiotic potential	High	Low	Moderate	Low

Table 3
Summary of biological mechanisms and apple pomace contributions.

Mechanism	Apple pomace contribution	Impact on cattle
VFA Production	High propionate and acetate from pectin/cellulose	Milk fat, energy metabolism
Rumen pH buffering	Increased chewing → more saliva → stable pH	Prevents acidosis
Microbial protein synthesis	Fermentable sugars support microbial growth	Milk protein, growth
Antioxidant modulation	Polyphenols reduce oxidative stress	Immunity, meat quality
Prebiotic effects	Pectin fosters beneficial microbes	Gut health, nutrient absorption

Additionally, polyphenolic compounds such as quercetin and chlorogenic acid may contribute to improved reproductive efficiency and immune modulation by reducing oxidative stress in high-producing dairy cows (Boyer and Liu, 2004; Fidriyanto *et al.*, 2023).

In beef cattle, inclusion of apple pomace up to 10% DM supports average daily gain (ADG) and feed conversion ratio (FCR), particularly when diets are balanced with energy-rich components such as cereal grains (Dai et al., 2022; Tiwari et al., 2008). The antioxidant properties of polyphenols enhance oxidative stability of muscle tissue, reducing lipid peroxidation and improving shelf life and sensory quality of beef (Fidriyanto *et al.*, 2023). Importantly, no adverse effects have been reported on dressing percentage or muscle-to-fat ratio at moderate inclusion levels. Balanced energy intake ensures optimal lean tissue deposition without excessive fat accumulation, making apple pomace a viable component in finishing rations (Gadulrab *et al.*, 2023).

Apple pomace also contains polyphenolic compounds such as quercetin, chlorogenic acid, and ursolic acid, which exhibit strong antioxidant and anti-inflammatory properties. These bioactives may reduce oxidative stress in high-producing dairy cows, particularly during early lactation when metabolic strain is elevated. By mitigating oxidative damage, these compounds support immune modulation and may enhance reproductive efficiency through improved ovarian function and reduced inflammatory cytokine activity (Boyer and Liu, 2004; Kruczek *et al.*, 2017; Ma *et al.*, 2022; Vendruscolo *et al.*, 2008). Recent studies have demonstrated that methanol and fermented alcohol extracts of dried apple pomace show high radical scavenging activity (DPPH and ABTS assays), with antioxidant capacity exceeding that of ascorbic acid in some cases. These findings suggest that apple pomace-derived antioxidants may play a supportive role in maintaining reproductive and metabolic health in dairy cattle under physiological stress (Boyer and Liu, 2004; Kruczek *et al.*, 2017; Ma *et al.*, 2022).

6. Economic Analysis

Apple pomace is often available at low or zero cost from juice processors, making it an economically attractive feed ingredient. Drying and processing costs typically range from 30 to 70 USD per ton, depending on the scale of operation and the technology used (Schmid *et al.*, 2020). Replacing 10–15% of conventional feed ingredients with apple pomace can reduce total feed costs by approximately 5–12%, particularly in regions with abundant apple production (Pascoalino *et al.*, 2025).

Beyond direct cost savings, apple pomace offers opportunities for local entrepreneurship in drying and pelletising operations. Its utilisation also reduces environmental disposal costs for juice producers and enhances feed self-sufficiency in apple-growing regions. These benefits align with circular economy principles and agro-industrial waste valorisation strategies (Pascoalino *et al.*, 2025).

Despite these advantages, several risks must be managed. Seasonal availability and variability in composition require flexible formulation strategies. Quality control measures, including mycotoxin monitoring and microbial safety checks, are essential to ensure feed safety. Furthermore, logistics and storage infrastructure must be optimised to handle dried pomace efficiently, preventing spoilage and maintaining nutritional quality (Piñeiro, 2008; Shalini and Gupta, 2010; Tulej and Głowacki, 2022).

7. Conclusions and Recommendations

Apple pomace-derived fibre is a nutritionally viable and economically sustainable feed ingredient for ruminants. Its inclusion in dairy and beef cattle diets supports rumen health, productivity, and meat quality while contributing to the valorisation of agro-industrial residues. To maximise its potential, future research should focus on standardising processing protocols to ensure consistent quality, evaluating long-term health and reproductive outcomes, and developing region-specific feeding guidelines based on pomace composition. Integrating apple pomace into certified organic feed systems and conducting life-cycle assessments will further strengthen its role in sustainable livestock production.

By addressing variability in composition, improving processing efficiency, and ensuring robust quality control, apple pomace can be transformed from a waste product into a strategic resource for sustainable ruminant nutrition.

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ORIGINAL ARTICLE

Molecular Detection of *Mycoplasma* in Milk Samples of Goat Herds in Borujerd County

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Abstract

Mycoplasmas are among the smallest bacteria which are responsible for economically significant infections in small ruminants. This study aimed to detect *Mycoplasma* in goat milk samples from Borujerd County, Iran. Ninety-six milk samples were collected from seven regions and screened for subclinical mastitis using the California Mastitis Test (CMT) and somatic cell count (SCC). Thereafter, total DNA was extracted, and *Mycoplasma* detection was performed by a PCR assay using genus-specific primers. High SCC and CMT scores were observed in 7.3% and 31.25% of samples, respectively. PCR analysis confirmed *Mycoplasma* infection in 5.2% (n=5) of the milk samples. The presence of *Mycoplasma* infection in goat herds in Borujerd county highlights the need for breeders and veterinary supervisors to take effective measures to control and prevent these infections. Besides, these findings suggest PCR assay as a rapid and reliable method for identifying *Mycoplasma* in milk samples.

1. Introduction

Goats are among the most important domestic ruminants, widely raised across the world, including many regions of Iran, for meat and milk production. Small size, adaptability, low nutritional demands, and resistance to adverse environmental conditions are those attributes that make goats especially valuable for rural and small-scale farmers. However, bacterial infectious diseases, particularly mycoplasma infections, have posed a significant threat to goat production (Zamiri and Heidari, 2006).

Mycoplasmas are small, pleomorphic bacteria that typically cause chronic infections (McGowin and Totten, 2017; Hajizadeh *et al.*, 2018). Contagious agalactia, a notable mycoplasma disease of small ruminants, presents with inflammation of the udder, joints, and conjunctiva, and is endemic in many Mediterranean countries (Jay and Tardy, 2019). In this case, *Mycoplasma agalactiae* is the primary causative agent, though other species including *M. mycoides*, *M. capricolum*, *M. putrefaciens* and *M. arginini* have also been implicated (Quinn *et al.*, 2011). Infected mammary glands may secrete purulent material, progressing to fibrosis, and permanent loss of milk production. Economic impacts include abortions, weak offspring, udder deformities, joint swelling, and blindness (Madanat *et al.*, 2001). These outcomes underscore the importance of accurate detection and control.

Traditional diagnosis of mycoplasma infections relies on clinical signs, bacteriological culture, and serology; however, the fastidious growth requirements of mycoplasmas limit these methods. Molecular techniques such as PCR offer rapid, sensitive, and specific detection (Templeton *et al.*, 2003).

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Table 1
Primer sequences used in *Mycoplasma* genus-specific PCR

Primer	Sequence 5'-3'	Amplicon size (bp)	Reference
GPF- Forward	GCTGGCTGTGTGCCTAATACA	1013	Lierz *et al.*, 2007
MGSO- Reverse	TGCACCATCTGTCACTCTGTTAACCTC		

Considering the importance of mycoplasma infections and the economic losses caused by these bacteria in goat herds, the present study was carried out to molecularly investigate mycoplasma infection in milk samples from goat herds in Borujerd county.

2. Materials and Methods

2.1. Sampling

Milk samples were collected from 96 clinically healthy goats in seven regions of Borujerd county (Shisheh, Shahviran, Jahanabad, Karvaneh, Asadkhani, Darehgorg, and Kenarvand) between 2018 and 2019. Sampling was performed seasonally: Shisheh and Shahviran (summer 2018), Jahanabad and Karvaneh (autumn 2018), Asadkhani and Darehgorg (winter 2018), and Kenarvand (spring 2019). Twenty-four samples were collected from Kenarvand, and 12 from each of the remaining regions.

To do sampling, teats were washed, disinfected with 70% ethanol for 30 s, and the first streams of milk were discarded. Approximately 10 mL of milk was collected from the third stream onward into sterile containers, labeled with animal data, and transported on ice to the Bacteriology Laboratory, Faculty of Veterinary Medicine, Bu-Ali Sina University. None of the animals had received antimicrobial treatment at the time of sampling.

2.2. California Mastitis Test (CMT)

Somatic cell levels were estimated using the California Mastitis Test (Ruegg, 2017). Equal volumes of milk and CMT reagent were mixed in test wells and reactions were scored within 10 s as negative, +1, +2, or +3 based on clot formation.

2.3. Somatic Cell Count (SCC)

SCC was determined using Giemsa staining. Ten microliters of milk samples were smeared over 1 cm^2 on a glass slide, air-dried, fixed in xylene (5 min) and ethanol (3 min), stained with Giemsa (1 min), and decolorized in absolute ethanol (5–10 s). Slides were then examined under a 40× light microscope, and counts from 10 fields were multiplied by 5×10^4 to yield SCC/mL. Samples with $>3\times10^5$ cells/mL were classified as high SCC (Dohoo *et al.*, 1984).

2.4. DNA Extraction

Total DNA was extracted from milk samples using a commercial kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. DNA quality was assessed by agarose gel electrophoresis and suitable samples were stored at −20°C until analysis.

2.5. PCR Detection of Mycoplasma

Genus-specific PCR targeting a conserved region of the *Mycoplasma* genome was performed using primers GPF and MGSO (Lierz *et al.*, 2007). Each 25 μL PCR reaction contained 12.5 μL master mix, 1 μL of each primer (10 pmol), 5 μL DNA template, and 5.5 μL sterile distilled water. Thermal conditions were as follow: 94 °C for 5 min; 40 cycles of 94 °C, 55 °C, and 72 °C for 1 min each; and a final extension at 72 °C for 10 min. DNA from a commercial contagious agalactia vaccine (Razi Vaccine and Serum Research Institute, Karaj, Iran) served as a positive control, and distilled water as a negative control. Amplicons were visualized on 1% agarose gels.

3. Results

3.1. California Mastitis Test

All 96 milk samples were evaluated by CMT and classified into four categories: negative, +1, +2, and +3. As shown in Table 2, 20% (n=19) of samples were negative, while 80% (n=77) were positive distributed across the +1 to +3 categories.

Table 2
Percentage frequency of milk samples in terms of score in CMT

CMT Scores	No. of samples (%)
Negative	17 (17.7)
+1	22 (22.91)
+2	27 (28.12)
+3	30 (31.25)

3.2. Somatic Cell Count

SCC was determined according to the Giemsa staining method (Table 3). Samples with SCC >300,000 cells/mL were classified as high-SCC. An example of Giemsa-stained somatic cells from a high-SCC sample is shown in the Fig. 1.

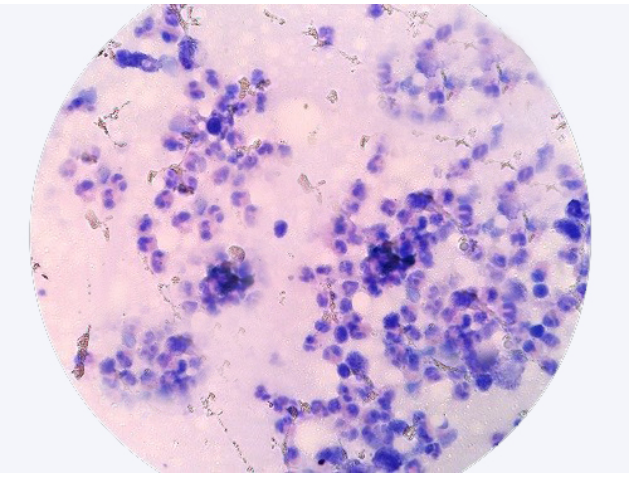

Fig. 1. Microscopic view of a Giemsa-stained smear for somatic cell counting (×40 magnification).

Table 3
Frequency distribution of milk samples in terms of somatic cell count.

Group	SCC	No. of samples (%)
A	$< 10^5$	50 (52.08)
B	10^5–2×10^5	29 (30.21)
C	2×10^5–3×10^5	10 (10.42)
D*	$3\times10^5 <$	7 (7.29)

D: Milk samples with high SCC

3.3. PCR Detection of Mycoplasma

PCR assay, as a molecular method with high sensitivity and specificity, was performed for molecular detection of *Mycoplasma* contamination in DNA samples extracted from goat milk samples. Positive samples yielded a 1,013 bp amplicon on agarose gel electrophoresis (Fig. 2). Totally, PCR results indicated that *Mycoplasma* DNA was present in 5 of 96 goat milk samples (5.2%). The characteristics of animals infected with *Mycoplasma* are presented in Table 4. As shown, *Mycoplasma* infection occurred in animals across different age groups.

4. Discussion

Mastitis, a common complication in cattle, has received less attention in small ruminants. However, given the large goat population in Iran and their considerable milk production, mastitis in goats warrants serious attention, as it can cause significant economic losses. Among the major causes of mastitis in goats is *Mycoplasma*, particularly *Mycoplasma agalactiae*, the causative agent of contagious agalactia which is a disease known for nearly two centuries. This disease is characterized by mastitis, arthritis, and keratoconjunctivitis (Kumar *et al.*, 2014; Quinn *et al.*, 2015). Although goats are generally less susceptible to mastitis than cattle, chronic, acute, and gangrenous forms can occur. Infections with *M. agalactiae* and *M. mycoides subsp. mycoides* often present with mastitis as a prominent symptom. Since mastitis is often subclinical, SCC and CMT tests are valuable for early detection. The CMT is a rapid, field-friendly test for estimating somatic cell counts, though in goats, high SCC values may occur in the absence of infection, limiting the test's diagnostic specificity. Nevertheless, increased SCC typically indicates mastitis (Contreras *et al.*, 2007).

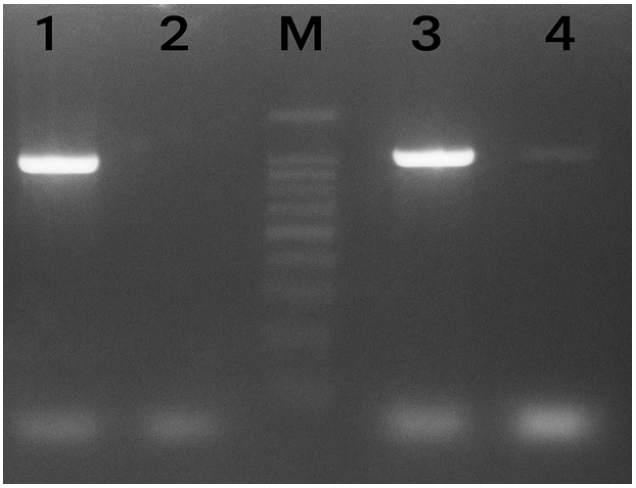

Fig. 2. Electrophoresis results of PCR products to identify genus *Mycoplasma*. Lanes 1 and 4: examined milk samples, lane 2: negative control, distilled water, lane 3: positive control, DNA extracted from the agalactia vaccine, lane M: a 100 bp DNA ladder.

In the present study, 96 goat milk samples from seven regions of Borujerd county were tested. In the CMT test, only 17 samples (17.7%) were negative; the remaining samples scored +1 to +3, with 30% scoring +3, indicating subclinical mastitis. In the SCC test, 7.3% of samples were in group D (>300,000 cells/mL). All high-SCC samples were also in the CMT 3+ group, confirming agreement between the two methods. Similar correlations between CMT and SCC have been reported by Persson and Olofsson (2011), who found both tests suitable for identifying mastitis in goats. In the evaluation of subclinical mastitis using SCC and CMT tests, all regions except Asadkhani had relatively similar conditions. No cases suspected of subclinical mastitis were found in Asadkhani (Table 4). Whereas, goats infected with *Mycoplasma* were detected only in the Shisheh and Jahanabad regions. All samples with high SCC values (except one) were classified in group D and corresponded to the CMT 3+ category. Furthermore, our regional analysis showed no subclinical mastitis in Asadkhani, maybe due to better hygiene and management practices.

Table 4
Number of milk samples with high SCC and CMT scores, and *Mycoplasma*-infected samples by region.

Sample collection area	No. of collected samples	No. of samples with high SCC	No. of samples with high CMT score	No. of *Mycoplasmainfected* samples
Shisheh	12	2	5	2
Shahviran	12	0	3	0
Jahanabad	12	1	5	2
Karvaneh	12	2	7	0
Asadkhani	12	0	0	0
Darehgorg	12	0	4	1
Kenarvand	24	2	6	0

Differences in SCC and CMT results between studies may be attributed to factors such as stress, breed, season, milking frequency, and infection type. Seasonal variation also influences SCC, with the highest counts from October to mid-February. Additionally, SCC accuracy can vary depending on whether automated or manual counting methods are used (Contreras *et al.*, 2007).

Sporadic reports of *Mycoplasma*-associated mastitis in Iran have emerged in recent years (Moradi *et al.*, 2011; Pourbakhsh *et al.*, 2013). Limited data exist on *Mycoplasma* infections in goat herds in Iran, as most studies focus on sheep due to their larger population. However, given that *M. agalactiae* causes severe milk yield losses and goats are a key dairy species, investigating its prevalence in goats is critical.

In this study, total DNA was extracted from milk samples using a commercial kit, and the *Mycoplasma* genus was detected by a PCR assay using specific primers. Five samples (5.2%) were positive. All of these *Mycoplasma*-positive goats were found only in Shisheh and Jahanabad.

Similar studies have reported varying prevalence rates across Iran, influenced by region-specific factors and diagnostic methods. However, PCR is generally more sensitive than culture (Moradi *et al.*, 2011; Mohammadpour *et al.*, 2015; Rahimabadi *et al.*, 2017).

Moradi *et al.* (2011) collected 367 milk samples from sheep and goats in Sanandaj and screened them for *Mycoplasma* contamination by culture on PPLO medium and by a PCR method using a specific primer pair for *Mycoplasma agalactiae*. Twelve samples (5.5%) were culture-positive, and five were PCR-positive for *M. agalactiae*, including one goat milk sample. Pourbakhsh *et al.* (2013) analyzed 142 sheep and 85 goat samples (joint fluid, eye secretions, and milk) using PCR. *Mycoplasma* was detected in 59 sheep (41%) and 46 goats (54%), of which 17 sheep (29%) and 28 goats (61%) were *M. agalactiae*. The authors reported a higher prevalence in goats and identified Kerman as a high-risk area due to large goat populations. In Ardabil, Hajizadeh *et al.* (2018) examined 116 sheep and 16 goats. Of the 132 animals tested, 33 (25%) were culture-positive in milk samples. PCR identified *M. agalactiae* in 47% of these, *Mycoplasma putrefaciens* in 43%, *Mycoplasma capricolum* in 7.5%, and *Mycoplasma mycoides* in 2%. The findings indicated that non-*agalactiae Mycoplasma* species are common in Iranian flocks and may be transmitted between sheep and goats. Mohammadpour *et al.* (2015) investigated 25 milk samples from five sheep flocks in Guilan. Culture identified nine positive samples (36%), while PCR detected 17 positives (68%), including five *M. agalactiae*. The study supported PCR as a reliable detection method as well. Shamsaddini *et al.* (2017) tested milk, joint fluid, and eye secretions from sheep and goats. Culture yielded 15% positivity, while PCR targeting the 16S rRNA gene detected *Mycoplasma* in 36% of samples. Rahimabadi *et al.* (2017) examined 71 samples (vaginal swabs, ear swabs, and milk) in Guilan. *Mycoplasma* colonies were observed in 50 samples (70.4%) by culture, while PCR confirmed *Mycoplasma* identity in 40 (80%) of these samples including 11 *M. agalactiae* (27.5%). Overall, prevalence rates vary widely by location, likely due to regional differences in management and environmental conditions. Most studies report PCR to be more sensitive and accurate than culture, though Tatay-Dualde *et al.* (2015) found both methods highly reliable for detecting *M. agalactiae* in goat milk and recommended culture of bulk-tank milk over PCR for routine surveillance. Another important aspect of *Mycoplasma* epidemiology is vertical transmission, which facilitates herd-level spread (De la Fe *et al.*, 2009). While semen samples were not tested in this study, future work should include semen and vaginal secretions to provide a more complete epidemiological picture. Previous research has found high *Mycoplasma* prevalence in goat semen using both culture and PCR, underscoring the importance of reproductive materials in disease transmission as well (Pourbakhsh *et al.*, 2017).

5. Conclusion

This study confirms the presence of *Mycoplasma* contamination in goat herds in Borujerd, albeit at a low prevalence. Nevertheless, the risk of transmission to other livestock, including sheep, warrants attention due to the potential for insidious reductions in herd productivity. The results also suggest the PCR assay as a rapid, sensitive, and reliable molecular tool for detection of *Mycoplasma*.

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REVIEW ARTICLE

Antibiotic Residues in Milk and Their Detection Methods: A Review

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Abstract

Milk and dairy products play a crucial role in human nutrition, but their quality can be affected by various factors, impacting marketability and consumption. Antibiotics are commonly used in the livestock industry for treatment and growth promotion. However, inadequate supervision and abuse by some producers, as well as improper drug use, can result in the excretion of drug and antibiotic residues into livestock products. These residues can then be secreted into milk and remain unchanged in the final product, potentially causing health problems for humans such as increased antibiotic resistance and hypersensitivity reactions. Therefore, it is essential to evaluate drug residues in milk and dairy products. Today, various methods are used to identify and quantify the amount of antibiotics in food. It is crucial for experts to choose a reliable and accessible method for this purpose. Implementing a dependable monitoring system, conducting regular inspections, and preventing excessive drug use are all vital steps in minimizing drug residues.

1. Introduction

Milk has a short shelf life because of its high nutritional content and the potential for contamination by various microorganisms like Staphylococcus, *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, etc. Therefore, temperature control is crucial from the milking process to storage in the raw milk tank and pasteurization process (Fusco *et al.*, 2020). Various chemicals from agricultural, veterinary, and sanitary pollutants may also enter milk. These substances include veterinary drugs such as antibiotics, hormones, anthelmintic, pesticides, and more (Khaniki, 2007).

Antibiotics are compounds produced from synthetic, semi-synthetic, or natural substances (Ahmed *et al.*, 2017). These compounds are mainly used to treat diseases and as food supplements. Additionally, they are administered orally or by injection to enhance the growth and increase the productivity of animals. If antibiotics are not used correctly, drug residues may remain in milk and affect the consumer (Schenck and Callery, 1998). More than half of the global production of antibiotics is used for animals (Girmatsion *et al.*, 2021), which can lead to increased resistance in microbes. Antibiotic residues may remain after treatment, so it is necessary to ensure that food from treated animals does not contain levels exceeding the standard (Vercelli *et al.*, 2023). The presence of antibiotic residues in milk may lead to resistance in microbes, a weakened immune system, allergic reactions, and even tissue damage and neurological disorders (Vercelli *et al.*, 2023). Some possible antibiotics found in

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milk include aminoglycosides, beta-lactams, tetracycline, macrolides, chloramphenicol, florfenicol and others.

1.1. Aminoglycosides

Aminoglycoside antibiotics consist of an aminoglycoside and an aminocyclitol ring linked by a glycosidic bond. These antibiotics include gentamicin, neomycin, dihydrostreptomycin, and streptomycin, and are primarily used in food-producing animals. Residues of aminoglycoside antibiotics, if present in milk, can damage the kidneys, cranial nerves, and lead to hearing loss. The FDA has set the tolerable levels of dihydrostreptomycin and neomycin in milk at 125 and 150 ng/mL, respectively, and the level of concern for gentamicin in milk at 30 ng/mL (Schenck and Callery, 1998).

1.2. Beta-Lactams

Beta-lactam antibiotics, such as penicillins and cephalosporins, are a concern due to the potential for causing allergic reactions in some individuals. In the United States, there are strict legal requirements regarding beta-lactam residues in milk, with all milk being mandated to undergo testing for these residues. The limits are set at 5 ng/ml for penicillin G, 10 ng/ml for amoxicillin, ampicillin, and cloxacillin, and 20 ng/ml for cefapirin (Schenck and Callery, 1998).

1.3. Tetracyclines

Tetracycline antibiotics are commonly used to treat bovine mastitis. The FDA has established levels of concern for residues of chlortetracycline, oxytetracycline, and tetracycline in milk at 30, 30, and 80 ng/mL, respectively (Olak *et al.*, 2007).

1.4. Macrolides

Macrolide antibiotics are more effective against gram-positive bacteria and are used to treat a wide range of infections. Pirlimycin hydrochloride is approved by the FDA for intramammary infusion treatment of clinical mastitis in dairy cows. A tolerance of 400 ng/mL of pirlimycin in milk has been established (Schenck and Callery, 1998).

1.5. Chloramphenicol, Florfenicol, and Thiamphenicol

These antibiotics have a broad spectrum and are suitable for treating a variety of infectious organisms. A small percentage of people exposed to chloramphenicol have been reported to develop aplastic anemia; the drug is not approved for use in food-producing animals in the United States (Schenck and Callery, 1998). Florfenicol is approved for the treatment of bovine respiratory disease in the United States. The FDA has set a level of concern for this drug in milk at 10 ng/mL (Schenck and Callery, 1998). Unlike many of the more polar antibiotics, the three antibiotics chloramphenicol, florfenicol, and thiamphenicol can be extracted from biological matrices using an organic solvent. A simple shake with ethyl acetate is sufficient to extract a small amount of chloramphenicol and florfenicol from milk (Wal *et al.*, 1980).

2. Methods for Identifying Antibiotic Residues in Milk

Due to the consequences of antibiotic residues, several laws and regulations have been enacted, including the development of methods for detecting antibiotic residues (Toldrá and Reig, 2006). Antibiotic residue detection methods are mainly divided into two groups: screening and confirmatory. Confirmatory assays are usually based on quantifying the concentration of the analyte (Cháfer-Pericás and Maquieira, 2010). Screening methods are used to determine if a sample contains antibiotic residues. If a positive result is obtained, an appropriate method is then used for confirmation, identification, and quantification. These methods detect an analyte at a concerning level and typically provide semi-quantitative results. Characteristics of these methods include lower false-positive rates, high throughput, simplicity, time-saving, low cost, and better selectivity (Liousia *et al.*, 2015). Methods commonly used for screening antibiotic residues include chromatographic, microbiological, and immunoassay methods (Yang *et al.*, 2014).

3. High-Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique that requires specialized equipment. This technique is time-consuming due to the preparation of samples and reagents and must be performed by skilled and trained personnel. Fat, protein, and sugars present in milk can compromise the correct identification of residues. HPLC is considered the gold standard method for detecting antibiotic residues due to its high sensitivity and quantitation (Vercelli *et al.*, 2023). The HPLC instrument consists of five main components: mobile phase, detector, pump, column, and sampler (manual or automatic). Samples are injected and transported through a column by the flow of the mobile phase. The pump generates the desired flow and pressure and pushes the mobile phase through the column to the detector. As a result, a signal is generated that is proportional to the amount of compounds present in the sample (Parmar *et al.*, 2021). The compound separated during HPLC can be identified, and its concentration in the sample calculated by comparing the peaks obtained from the analysis with a calibration (reference) curve

(Vercelli *et al.*, 2023). The use of HPLC for the detection of tetracycline, sulfonamides, and chloramphenicols in milk has been validated. This method was used to analyze 128 raw bovine milk samples, and oxytetracycline residues were detected in 4.9% of the samples, while amoxicillin residues were detected in 6.1% of the samples, maintaining the same performance reported in the initial phase. Kumar *et al.* (2022) performed the same method for pasteurized milk samples, but amoxicillin was not detected, possibly due to heat treatment or dilution of the samples (Kumar *et al.*, 2022).

4. Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

LC-MS/MS is an advanced analytical technique that excels in identifying and quantifying antimicrobial drugs, their metabolites, or residues in food like milk. These residues are characterized with very high accuracy (Vercelli *et al.*, 2023). In this method, sample compounds are ionized, evaporated after being removed from the column, and then fragmented. They are separated based on their mass-to-charge ratio (m/z) in a mass analyzer. The detector measures the frequency or intensity of each ion with a different m/z value, which is proportional to the concentration of the analyte in the sample. Ions are typically very specific to a particular substance, allowing for precise identification and quantification. This technique is highly accurate, more so than HPLC, and is considered a high-standard and validated tool. The high accuracy of this technique enables the identification and quantification of very low levels. Additionally, it has the ability to detect multiple residues from different antibiotic classes simultaneously. The main disadvantages of this technique are its high cost, due to the purchase of equipment, long operating time, and the need to work under standardized experimental conditions (e.g., control of ionization, pH, analyte stability, etc.). Furthermore, the complexity of milk composition may lead to misleading results (Li *et al.*, 2017).

5. Ultra-High Performance Liquid Chromatography (UPLC)

It is an advanced technique that has been successfully applied for the detection of antimicrobial residues. It is used alone or in combination with electrospray ionization (ESI) and tandem mass spectrometry (UPLC-ESI-MS/MS) (Vercelli *et al.*, 2023). Recently, a study focused on the simultaneous detection of 38 veterinary antibiotic residues in raw milk using UPLC-MS/MS. The results showed that the variable recovery was 68–118% for drugs belonging to the β-lactam group, 79–118% for quinolones, 71–106% for sulfonamides, 76–116% for tetracyclines, 78–106% for macrolides, and 88–103% for lincosamides, with coefficients of variation less than 15% for day-to-day accuracy (Vercelli *et al.*, 2023).

6. Square Wave Voltammetry (SWV)

In SWV, a series of equal amplitude pulses are applied. In each forward pulse, chemicals are diffused to the electrode surface and immediately reduced or oxidized. During the reverse pulse, the recently oxidized or reduced chemical species return to their initial state in a reversible reaction. If the system is irreversible, no reaction occurs. Therefore, the current values just before and at the end of each pulse are measured, and the net or resultant currents are plotted as a function of the corresponding potential in a staircase waveform, resulting in a Gaussian signal. The resulting signals are larger than the forward and reverse signals due to the significant reduction of capacitive currents. This provides high sensitivity and better selectivity than other electroanalytical techniques. Additionally, this technique offers the advantages of rapid analysis, good analytical frequency, low cost, and ease of operation (Megale and Souza, 2023).

Due to the presence of functional groups such as amide, amine, aromatic rings, carbonyl, double bonds, triple bonds, and sulfur bonds in antibiotics and other pharmaceutical compounds, they can be identified using the SWV technique. These compounds can be oxidized or reduced, providing a measurable electrical signal at a specific potential. This allows for both qualitative and quantitative analysis. Quinolones and fluoroquinolones contain a central amine group in their structure with a non-bonding electron that acts as a donor and can be oxidized electrochemically. The values obtained from this process are directly proportional to the antibiotic concentration. The oxidation reaction occurs at the secondary amine group in these antibiotics, leading to the formation of hydroxylamine. Cephalosporins, a class of β-lactam antibiotics, are chemically and electrochemically active due to their β-lactam ring structure. They can easily undergo hydrolysis in the environment or in an electrochemical cell, with peak currents reflecting the concentration of cephalosporins. Similarly, penicillins also contain a β-lactam ring that can undergo irreversible electron transfer through electrochemical oxidation. Peak currents and potentials can be utilized for the quantification and identification of penicillin antibiotics in food samples (Zhu *et al.*, 2023; Baezzat *et al.*, 2021).

7. Microbiological tests

In this method, there is no need for sample preparation. When an inhibitory zone appears in the reference bacterial cultures, the milk sample is considered positive. The diameter of the inhibitory zone must

be measured to interpret the results (Vercelli *et al.*, 2023). This technique has low specificity, which may lead to false results. High somatic cells or pH changes in the case of mastitis may result in false negative results. Additionally, the amount of antibiotic residues cannot be quantified with this technique. Moreover, only a small number of antibiotic families can be identified using this method. However, this method is still the focus of numerous studies aimed at improving the current method due to its short execution time, versatility with different animal origin products (such as eggs, honey, raw, pasteurized, and bulk milk), and low costs (Vercelli *et al.*, 2023). The development of this technique has enabled the design of specific bacteria, such as Geobacillus stearothermophilus, Bacillus megaterium, and Bacillus licheniformis, for the detection of beta-lactams, macrolides, tetracyclines, quinolones, and sulfonamides, respectively. These bacteria can detect these substances at levels close to the maximum residue limits (MRL) (Vercelli *et al.*, 2023). In this technique, all the elements are placed in a test tube and milk is added to it. The tube is then incubated at the appropriate temperature to provide conditions for bacterial growth. In the absence of antibiotic residues, changes in turbidity and color due to the acidification of the pH of the medium, which indicate the normal growth of bacteria, can be observed visually. For example, there is a change in color of the medium from yellow to black for Geobacillus stearothermophilus and from pink to blue for Bacillus licheniformis. In the presence of antibiotic residues in the milk, bacterial growth is inhibited and no changes are visible. A similar method based on the growth of Bacillus subtilis is able to detect the presence of sulfonamides (Nagel *et al.*, 2013). Several kits based on this technique are now commercially available. Two of the most popular are: (a) the Dulcotest ST-NP, which can detect beta-lactams, aminoglycosides, macrolides, sulfonamides, tetracyclines, and diaminopyrimidines, and (b) the Charm® QUAD1 test, which only detects beta-lactams, quinolones, sulfonamides, and tetracyclines. Both tests are capable of detecting residues at the minimum MRLs and report a sensitivity of 95% (Vercelli *et al.*, 2023).

7.1. Optical Biosensors

Optical biosensors are a prominent and widely used type of sensor, prepared by integrating a biological detection element with an optical transducer. These devices operate based on optical transmission, collecting signals from one or more analytes to quantify crucial details in optical radiation, including frequency, amplitude, and polarization (Dezhakam *et al.*, 2024). The advantages of these biosensors include remarkable sensitivity and accuracy, the ability to detect a specific analyte, low background noise, satisfactory limit of detection (LOD), and rapid analysis. These biosensors are more cost-effective due to the support of multiple different biosensors with a fixed hardware system, along with a complementary metal oxide semiconductor (CMOS) camera. Additionally, optical biosensors are easy to transport and allow for remote detection to prevent contamination of the device by the analyte. This feature makes them a viable option for observing biological samples (Dezhakam *et al.*, 2024). Using optical biosensors and sensors, a variety of analytes such as proteins, DNA/RNA derivatives, bacteria, viruses, tissues, and drugs can be detected at low concentrations. Similar to electrochemical biosensors, nanoparticles such as AuNPs can be utilized in these biosensors to amplify the sensitivity of optical signals. Optical biosensors can be classified into label-free and label-based detection methods (Dezhakam *et al.*, 2024). In the label-free system, signals are generated as a result of the direct interaction between the transducer and the sensor analyte, without the presence of any conjugated label. In the label-based method, special materials such as fluorescent, colorimetric, and isotopic labels are used to obtain optical parameters. This method is more expensive and specialized compared to the label-free options (Khansili *et al.*, 2018).

Various optical biosensing techniques have been characterized to date, including fluorescence, colorimetry, spectrofluorometry, surface plasmon resonance (SPR), and Surface-Enhanced Raman Spectroscopy (SERS). Altintas utilized a combination of nanomolecularly imprinted polymer and surface plasmon resonance (SPR) techniques in his sensor structure for the detection of glycopeptide antibiotics, such as vancomycin, in milk samples (Dezhakam *et al.*, 2024).

The SPR technique is inspired by the phenomenon of surface plasmon. Plasmon refers to the oscillations of electrons in a metallic conductor when high-energy electrons pass through it. In this technique, polarized light is incident at a certain angle on the surface of a conductive material such as metal at the interface of two media (generally glass and liquid). This interaction between free electron density fluctuations and electromagnetic waves between the surface of the dielectric film and the metal leads to the generation of surface plasmon. By measuring the change in angle, reflection, or wavelength over time, various details about the concentration, equilibrium, and movement of molecular interactions can be obtained (Dezhakam *et al.*, 2024).

8. Immunological Methods

8.1. ELISA

The enzyme-linked immunosorbent assay (ELISA) technique was first described by Engvall, Johnson, and Perlman in 1971. It is based on antigen-antibody binding, which produces a colorimetric reaction due

to the presence of a chromophore bound to the antigen. The ELISA technique can be designed as qualitative (positive/negative result) or quantitative (result as a measurement or concentration). There is also a semi-quantitative test that provides different levels of positivity and negativity that must be compared with a reference scale. Several commercially available ELISA-based kits are used for the rapid detection of various families of antibiotics in bovine milk. These kits can be used as a screening test by veterinarians and dairy industry personnel to examine the milk of one or more cows due to their ease of implementation and low cost (Dezhakam *et al.*, 2024). If the ELISA test result is positive (meaning residues above the MRL are detected), it is necessary to perform a confirmatory test using more specific and sensitive tests such as high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS/MS). The ELISA technique has recently been validated for the detection of β-lactams in milk (Vercelli *et al.*, 2023).

A new technique based on magnetic immunoreactivity, inspired by antigen-antibody interaction, has been developed for the detection of kanamycin and penicillin in dairy milk. This technique shows very low limit of detection (LOD) and high sensitivity. Pretreatment and dilution steps of milk samples are necessary for this technique. However, it is important to note that this technique is still in the proof of concept stage and has not yet been commercialized (Schenck and Callery, 1998). An ELISA-based microarray assay has recently been approved under specific European regulations to simultaneously detect norfloxacin, tetracycline, lincomycin, and streptomycin in milk samples, achieving very high accuracy (ranging from 77.6% to 116.4% for different antibiotics). Its disadvantage is that it requires specialized and trained personnel (Vercelli *et al.*, 2023). So far, this method has been proposed for control programs in the dairy industry (Du *et al.*, 2019).

8.2. Lateral Flow Immunoassay (LFIA)

This technique, due to its simpler sample preparation, requires less time compared to ELISA and allows for the analysis of large amounts of samples with a low investment cost, providing rapid results. In recent years, this technique has been widely used for the detection of antibiotics in milk, such as beta-lactams, tetracyclines, streptomycin, and chloramphenicol (Vercelli *et al.*, 2023).

8.3. Radioimmunoassay (RIA)

Radioimmunoassays have been widely used in the past for detecting antibiotic residues in food because of their rapid performance and low limit of detection (LOD). However, the use of this technique has been limited to only a few clinical purposes due to the short half-life of the radioisotopes used to label the analyte and the potential hazards of using radioisotopes for personnel and the environment (Vercelli *et al.*, 2023).

8.4. Fluorescence Immunoassay

This method is based on the binding of a fluorophore to the antigen, and its detection is accurate and reliable. However, background signal can interfere with the emission, potentially leading to ambiguous results (Schenck and Callery, 1998). This method has been widely used for the detection of fluoroquinolones, beta-lactams, and tetracyclines in milk (Vercelli *et al.*, 2023).

9. Conclusion

Given the adverse effects of antibiotic residues on health and the development of antibiotic resistance, it is necessary to detect these residues in food using effective methods. The methods mentioned in this article are desirable for detecting antibiotic residues in milk. It is hoped that in the near future, new and more effective methods for detecting antibiotic residues in food and other areas will be presented.

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