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Metamizole Reduces The Expression of OCT4, SOX2, SOX4 in Hematopoietic Stem/Progenitor Cells Directed to Differentiation in In Vitro Hematopoiesis Simulation

Metamizol İn Vitro Hematopoez Simülasyonunda Farklılaşmaya Yönlendirilen Hematopoetik Kök/Progenitör Hücrelerin OCT4, SOX2, SOX4 Ekspresyonunu Azaltır

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ABSTRACT

Aim: Metamizole is a non-steroidal anti-inflammatory drug used for its analgesic and antipyretic effects. Metamizole-associated agranulocytosis is a significant adverse effect limiting its use in some countries, but it has also been shown to promote apoptosis in cancer cells with possible mechanisms underlying this adverse effect. The mechanisms underlying these effects have not been sufficiently elucidated.

Methods: The cells obtained from stem cell product samples collected from healthy allogeneic stem cell donors were used in the study. MTT assay was used to analyse the proliferative-cytotoxic effects of metamizole on cells. CFU-Mix culture was also performed for cell differentiation to granulocyte and analysis of expression of OCT4, SOX2, SOX4. The expression fold change of transcription factors was determined by RT-qPCR.

Results: Metamizole limited the proliferation of cells at increasing concentrations starting at 10 µM. The IC50 values of metamizole were significantly different between the samples. The lowest value for IC50 was 50.96±7.60 µM and the highest value was 165.5±18.21 µM. Granulocyte-macrophage colony counts and OCT4, SOX4 mRNA expressions decreased at metamizole concentrations of 10 and 100 µM. For SOX2, the decrease occurred at a concentration of 100 µM.

Conclusion: Our results indicated that metamizole may limit the proliferation and differentiation of hematopoietic stem/progenitor cells via transcription factors and this effect may be responsible for possible agranulocytosis cases. NSAIDs have also been shown to have apoptotic effects on cancer cells. The ability to limit the expression of transcription factors that play important roles in cell development and differentiation may pave the way for the synergistic use of this drug with antineoplastic drugs.

Keywords: Metamizole, NSAID, Hematopoesis, OCT4, SOX2, SOX4

ÖZ

Amaç: Metamizol analjezik ve antipiretik etkisi nedeniyle kullanılan bir non-steroidal antiinflamatuvar ilaçtır. Agranülositoz bu ilacın bazı ülkelerde kullanımını sınırlayan önemli bir yan tesiridir fakat bu yan tesirin olası mekanizmalarıyla kanser hücrelerinde apoptozu teşvik ettiği de gösterilmiştir. Bu etkilerin altında yatan mekanizmalar yeterince açıklanmamıştır.

Yöntem: Çalışmada sağlıklı allojeneik kök hücre donörlerinden toplanan kök hücre ürün örneklerinden elde edilen hücreler kullanıldı. Metamizolün hücreler üzerindeki proliferatif-sitotoksik etkilerinin analizi için MTT testi uygulandı. Ayrıca farklılaşmanın ve OCT4, SOX2, SOX4'ün ekspresyon analizleri için CFU-Mix kültürü yapıldı. Transkripsiyon faktörlerinin ekspresyon katsayıları RT-qPCR ile tespit edildi.

Bulgular: Metamizol 10 µM başlayarak artan konsantrasyonlarda hücrelerin çoğalmasını sınırlandırdı. Metamizolün IC50 değerleri örnekler arasında belirgin derecede farklıydı. IC50 için en düşük değer 50,96±7,60 µM en yüksek 165,5±18,21 µM olarak elde edildi. Granulosit-makrofaj koloni sayımlarında ve OCT4, SOX4 mRNA ekspresyonlarında 10 ve 100 µM konsantrasyonlarda azalma oldu. SOX2 için azalma 100 µM konsantrasyonda gerçekleşti.

Sonuç: Sonuçlarımız metamizolün transkripsiyon faktörleri üzerinden hematopoetik kök/progenitör hücrelerin proliferasyon ve farklılaşmasını sınırlandırabileceğini ve olası agranülositoz olgularında bu etkinin de sorumlu olabileceğini işaret etti. NSAII'erin kanser hücreleri üzerine de apoptotik etkileri de gösterilmiştir. Hücrelerin gelişmesi ve farklılaşmasında önemli rolleri olan transkripsiyon faktörlerinin ekspresyonunu sınırlandırabilmesi, bu ilacın antineoplastik ilaçlarla sinerjik etki için birlikte kullanımlarının önünü açabilir.

Anahtar kelimeler: Metamizol, NSAİİ, Hematopoez, OCT4, SOX2, SOX4

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Introduction

Metamizole, which is among non-steroidal anti-inflammatory drugs (NSAIDs), is prescribed for the treatment of moderate and severe pain and for its antipyretic effect. Metamizole may rarely cause agranulocytosis, which is characterized by a peripheral blood neutrophil count of less than 500 per microlitre. Cytotoxic and immunological mechanisms can be involved in its adverse effects. However, the mechanism of agranulocytosis has not yet been fully elucidated [1,2]. The adverse effects of metamizole on the hematopoietic system are generally based on case reports and reviews of case reports. These studies generally indicate that the risk may increase with long-term use of the pharmaceutical [3,4]. Experimental studies investigating the mechanisms of action on hematopoiesis are too limited. Studies using hematopoietic cell lines and mononuclear cells derived from transplantation samples suggest that metamizole can limit cell proliferation and granulocyte differentiation and inhibit Hematopoiesis [5,6,7]. Hematopoietic stem/progenitor cells can be isolated from samples such as cord blood, bone marrow and cultured in a semi-solid medium containing cytokines, growth factors simulating Hematopoiesis. In this culture called 'colony forming unit' (CFU) assay, in which the colony forming capacity of cells can be evaluated, differentiation and proliferation patterns can be examined [8].

Transcription factors act as molecules responsible for metabolism, development and differentiation in cells and play important roles in the synthesis of proteins. Some studies show associations of NSAIDs with transcription factors, but the designs of the studies are usually related to the mode of action of these pharmaceuticals. The peroxisome proliferator-activated receptor (PPAR) family includes three transcription factors and NSAIDs have been reported to be agonists for these [9]. NF-κB is an important molecule that transcribes a number of proinflammatory cytokines and anti-apoptotic proteins. In the early stages of experimental colorectal cancer, it has been found that the expression of inflammatory molecules mediated by NF-κB can be reduced by NSAID administration [10]. However, there is too limited knowledge about the contribution of transcription factors to the adverse effects of these pharmaceuticals. In addition, the effects of metamizole on OCT-4 (octamer-binding transcription factor 4), SOX-2 (Sex determining region Y-box 2) and SOX-4 (Sex determining region Y-box 4) have not been analysed. OCT4 (also known as OCT3 or OCT3/4) is encoded by the Pou5f1 (POU domain, class 5, transcription factor 1) gene. SOX2 is a member of the SRY (Sex-determining Region Y gene) related gene family. OCT4 and SOX2 have aroused great interest because blocking their expression in pluripotent stem cells and in the early stages of embryogenesis leads to serious developmental consequences. There is some evidence showing that these two transcription factors work together [11]. SOX4 has been found to have important roles in the development of blood cell types and B lymphocytes [12]. The adverse effects affecting cell proliferation and differentiation such as agranulocytosis caused by NSAIDs and some other pharmaceuticals indicate that the interactions between COX inhibition and transcription factors and between drugs and transcription factors should be investigated. Although there are no results related to metamizole, acetyl salicylic acid was analysed in planaria within the scope of STAT3, SOX2, OCT4 [13]. It is suggested that metamizole may have antidepressant-like [14] and neuroendocrine [15] effects and can stimulate apoptosis in some cancer cell lines [16,17]. Experimental studies to explain the mechanisms related to the adverse effects of metamizole, which is used for its strong antipyretic and analgesic effects, are very limited. It has been previously shown that metamizole may limit the proliferation and differentiation of hematopoietic stem/progenitor cells. In this study, we aimed to reveal the role of transcription factors in the effects of the drug on the proliferation and differentiation of these cells. Our results also show that metamizole and other similar drugs should be investigated in terms of these factors in their proliferation-limiting effects on cancer cells.

Materials and Methods

Hematopoietic stem/progenitor cell samples

Hematopoietic stem/progenitor cells product samples (n=10) were obtained from donors of

the patients who applied to Başkent University Adana Adult Bone Marrow Transplantation and Cellular Therapy Center and who were decided to undergo transplantation. These samples are representative of the stem cell product and are sent to the laboratory to determine the product quality, and the remaining amount after analysis is evaluated as medical waste. Mononuclear cells were isolated by density gradient method from the samples obtained with informed consent from donors who fully met the criteria for being a healthy donor. These cells were stored in the freezer and cultured by thawing at the time of the study. Healthy donor criteria basically include people who do not have any known genetic or chronic diseases, who do not use medication for any other reason, and whose laboratory results support their health in the analyses performed for donor screening.

Proliferation/Cytotoxicity analysis by cell culture – MTT

In the first stage of our study, proliferative/cytotoxic effects and the half maximal inhibitory concentration (IC50) of metamizole were determined by performing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich Corporation, UK) assay on the cells. For this study, 1x104 cells were seeded into each well of the 96-well plate to be analyzed in RPMI-1640 media (Thermofisher, Gibco, UK) supplemented with fetal bovine serum and antibiotics. Metamizole (Sigma Aldrich, USA) concentrations of 1-10-100-200 µM were used for proliferation/ cytotoxicity analysis. These concentrations were preferred based on literature information and our previous experience. In addition, a control group without active substance was created. At the 48th hour of culture, MTT solution was prepared and added into each well in a 96-well plate and left in incubation for 4 hours. The formed formazan crystals were dissolved with dimethyl sulfoxide (WAK-Chemie, Germany) and the plate was analyzed in a multimode reader (BioTek Synergy H1, USA) at 570 nm light wavelength with 630 nm wavelength reference to obtain absorbance values. MTT studies were performed in duplicate for each sample and in 8 wells for each group created from this sample.

CFU-Mix - Cell culture and RT-qPCR analysis of transcription factors

In the CFU-Mix Assay, colonies can be easily distinguished morphologically under the microscope. The granulocyte-macrophage colonies (CFU-GM) are a heterogeneous mixture of small round granulocytes and larger oval macrophage precursor cells, containing at least 40 cells. The cells are transparent, may be in the thousands, and appear to cluster around a center. To perform CFU-Mix culture of cells, semi-solid medium (MethoCult4034, Stem Cell Technologies, Canada) containing cytokines that provide the development of erythroid, granulocyte, macrophage and megakaryocyte colonies was used. The culture was performed in duplicate with a total of 5x105 cells in each of 35 mm diameter petri dishes. In this culture method, groups were formed for 1-10-100 µM concentrations of metamizole together with the substance-free control group. Based on our previous experience and provided results of MTT assay, 200 µM concentration was not used in these studies because the drug reduces proliferation to a high extent. The CFU-GM formed in the petri dish were counted at the end of 14 days of culture. The colonies in petri dishes were counted and noted in duplicate by two researchers. The number of colonies in the control group of each sample was matched to 100% and the colony rates of the other groups were calculated accordingly. The cells were collected from the petri dishes after counting colonies and total RNA was extracted with a ready-to-use commercial kit (Norgen Biotek, Canada) for PCR analysis. After measuring the concentrations of these RNA samples, complementary DNA samples were obtained in the thermal cycler device (Applied Biosystems, SimpliAmp, Singapore) with the commercial kit (ABM, USA). Threshold cycle (CT) values were obtained after RT-qPCR analysis was completed in the Light Cycler 96 device (Roche, Germany). Duplicate studies were performed for each primer in RT-qPCR analyses with cDNA samples obtained from cells taken from petri dishes. Beta-actin was used as a housekeeping gene for the analysis. Information about the primers is given in Table 1.

Table 1: The primers gene IDs and reference positions used in RT-qPCR analysis.

Gene (Qiagen,Germany)		Gene ID	Reference Position	Qiagen -GeneGlobe ID
OCT4 (POU5F1)	POU class 5 homeobox 1	5460	NM_002701	PPH02394E
SOX4	SRY (sex determining region Y)-box 4	6659	NM_003107	PPH01950A
SOX2	SRY (sex determining region Y)-box 2	6657	NM_003106	PPH02471A
β-Actin	Actin, beta	60	NM_001101	PPH00073G

Statistical analysis

The absorbance values obtained for MTT analysis in our studies were transformed into percentage values. Similarly, colony counts were also transformed to percentage values since each product sample can form different total number of colonies. The CT values of control groups obtained in RT-qPCR were normalized according to the reference (housekeeping gene) β-Actin and fold change [2(-ΔΔCT)] calculations were performed. The control group's fold change values were transformed to 1. Calculations of the other groups were made according to the control. The data obtained from the laboratory studies were analyzed with GraphPad Prism version 9.0.0 (San Diego, CA, USA) software and presented as mean ± SD. Data distributions were tested for normality using the Shapiro-Wilk test. As data were not normally distributed, Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test was used to analyse the data obtained. p values less than 0.05 were considered significant. In addition, increases in relative expression results greater than two-fold compared to the control were considered physiologically significant.

Results

The proliferation/viability culture analysis for 48-hour, and the culture studies for 14-days simulating hematopoiesis in vitro for analysis of transcription factors were successfully completed with hematopoietic stem cell product samples.

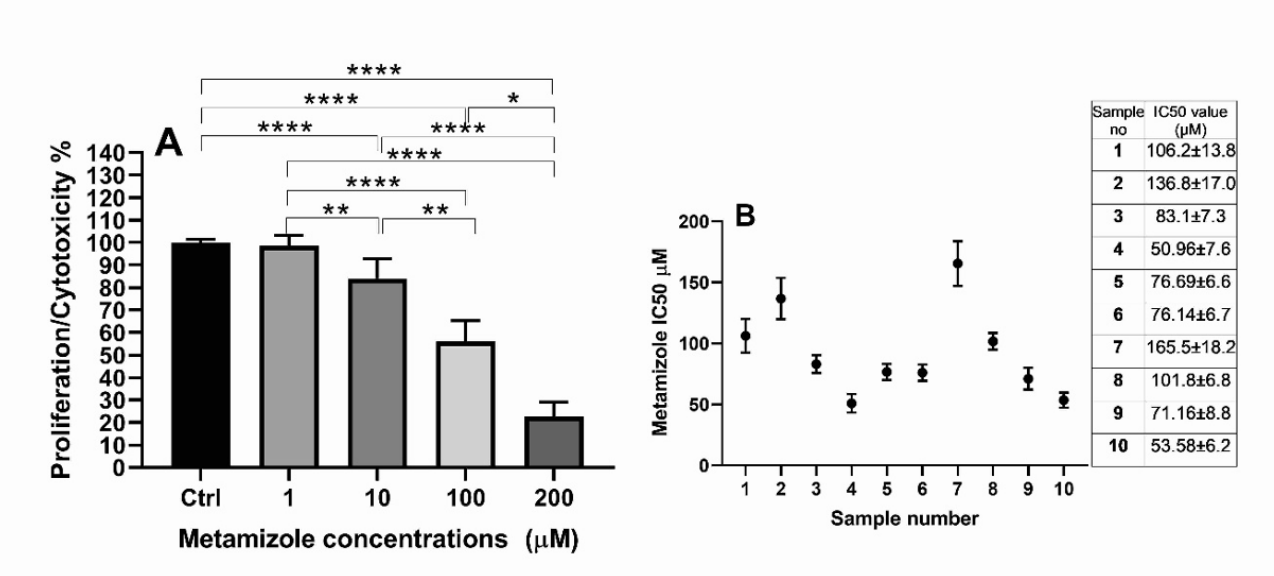
Proliferation/Cytotoxicity analysis-MTT

According to MTT analysis, our results showed that metamizole significantly affected and limited the proliferation of cells starting from 10 µM concentration (Figure 1A). The cell viability was measured as 100±1.45% for control group and 98±4.64% - 84.04±8.81% - 56.1±9.17% - 22.64±6.45% for metamizole groups at 1-10-100-200 µM concentration, respectively. However, the average IC50 value was obtained as 92.19±36.31 µM. The IC50 value was analysed for each sample separately and showed individual differences in behaviour towards the effect of metamizole. The highest IC50 value was 165.5±18.21 (sample 7) and the lowest was 50.96±7.60 µM (sample 4). The IC50 distribution between the samples is shown in Figure 1B.

Colony counting and RT-qPCR analysis of transcription factors

Colony growth in our CFU-Mix culture samples was monitored microscopically until day 14. In terms of colony appearance, especially the views of GM colonies were obtained in a way that was compatible with healthy product samples (Figure 2). Small numbers of GEMM (granulocyte, erythroid, macrophage, megakaryocyte) colonies were also counted. Similar to our MTT results, it was determined that metamizole limited the increase of GM colonies starting from 10 µM concentration in our methylcellulose-based CFU-Mix culture results as well. The significant differences were observed in all groups except 1 µM compared to control ($p<0.0001$). While the GM colony capacity was obtained as 100±8.02% for the control group, it was determined as 97.72±9.93%, 79±11.33%, 49.17±15.81% for the 1-10-100 µM groups (Figure 3).

Our results determined by RT-qPCR analysis show that OCT4 and SOX4 expressions are reduced at 10 and 100 µM concentrations. An important finding we observed in our studies is that SOX2 expression in these cells is not at a sufficient level to be analyzed by RT-qPCR. SOX2 expression was reliably detected in only 4 samples. Our studies were performed in 36 cycles, but for SOX2, it was increased up to 50 cycles. However, since the reliability decreases with further cycles and the deviations between the results are too large, the result analyses were performed with samples with CT data up to 36 cycles. No results were obtained



Sample no	IC50 value (μM)
1	106.2±13.8
2	136.8±17.0
3	83.1±7.3
4	50.96±7.6
5	76.69±6.6
6	76.14±6.7
7	165.5±18.2
8	101.8±6.8
9	71.16±8.8
10	53.58±6.2

Figure 1. A: The changes in the proliferation/cytotoxicity ratio of cells at increasing concentrations of metamizole. B: The IC50 concentrations obtained in each individual sample. The table shows the mean IC50 value of the samples in the graph. Kruskal Wallis and Dunn's multiple comparison. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001

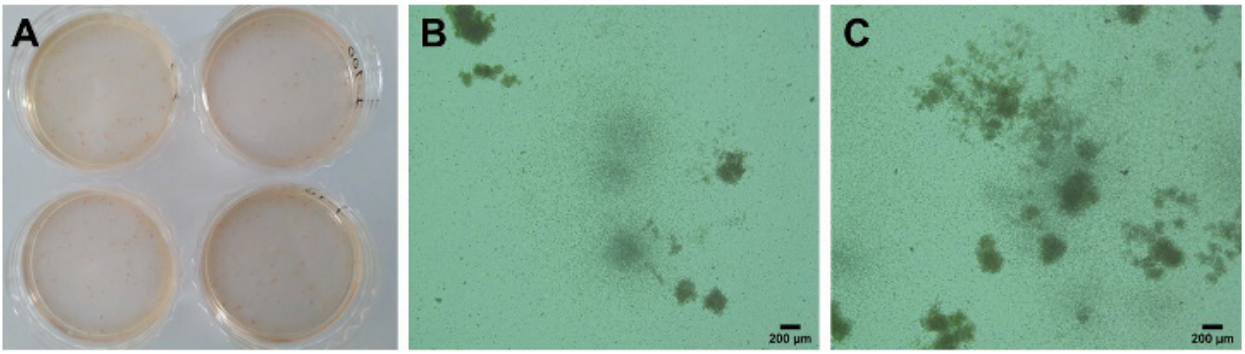


Figure 2. A: Macroscopic (in petri dishes) and B,C: microscopic views of the colonies.

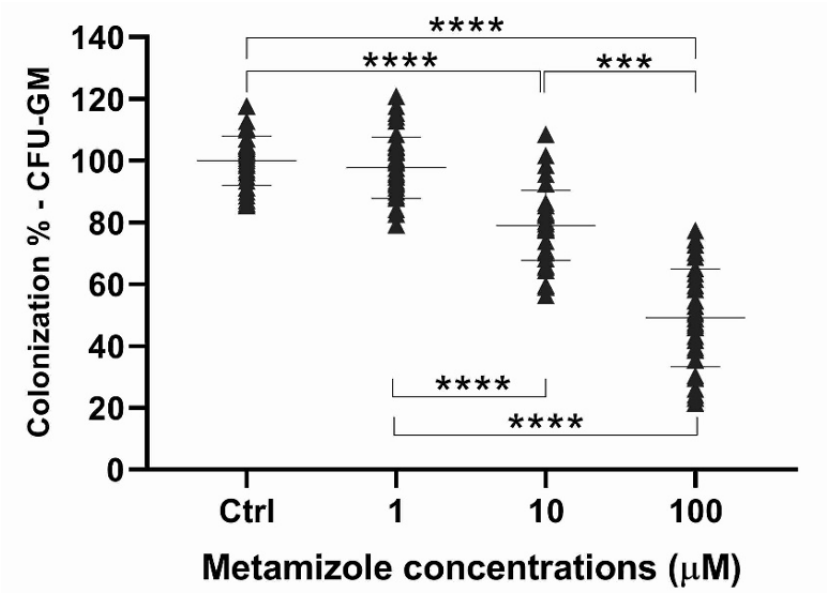


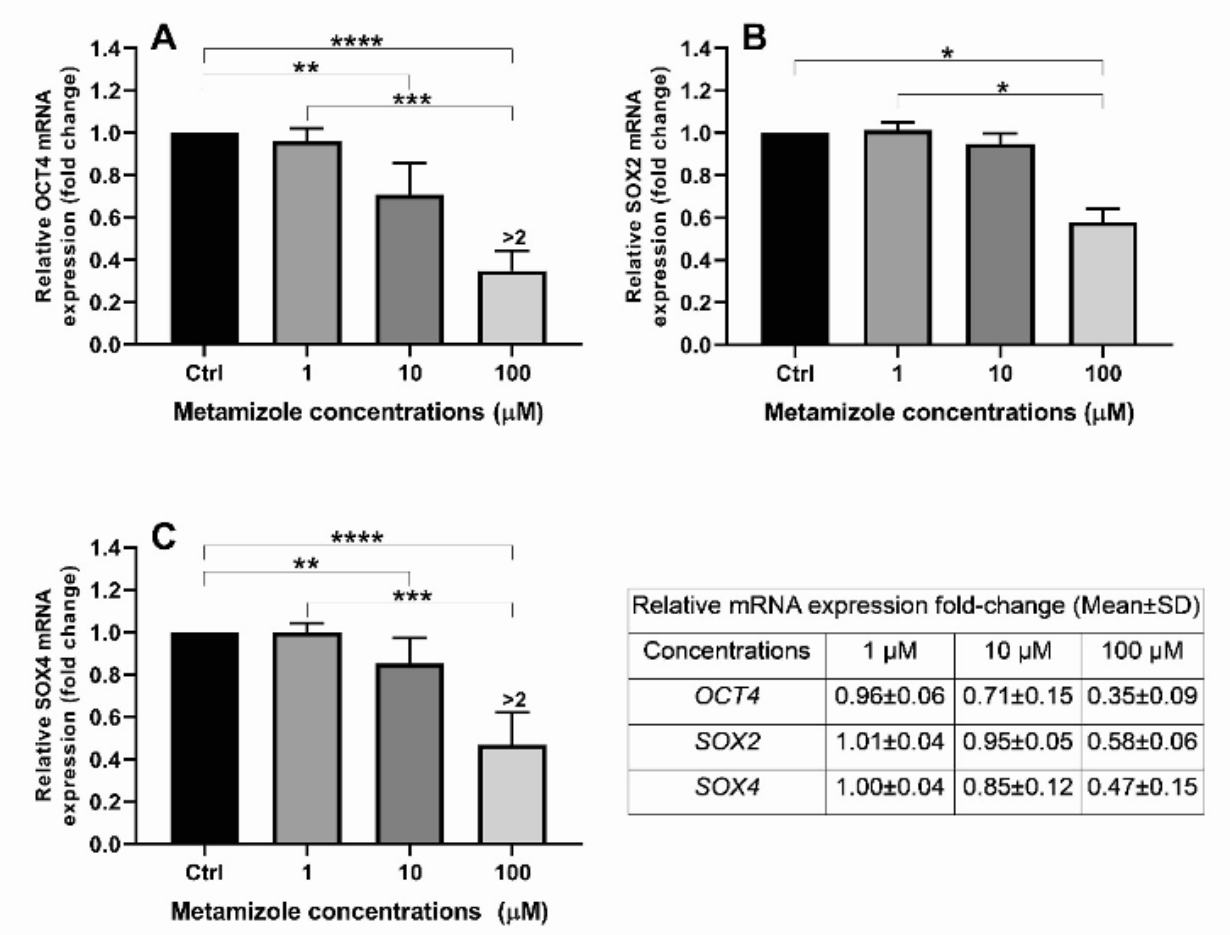
Figure 3: Showing decreasing number of CFU-GM colonies against increasing concentrations of metamizole. Kruskal Wallis and Dunn's multiple comparison. ***p<0.001, ****p<0.0001

from 4 samples (the experiment was repeated for these samples). According to these data, a significant decrease was detected only at 100 µM concentration compared to both control and 1 µM (Figure 4).

Discussion

Some countries have restricted the use of metamizole due to its rare but serious adverse effects on hematopoiesis. This limitation, especially in developed countries, has caused the pharmaceutical to be studied less than other NSAIDs in different indications [18]. Serious adverse effects of metamizole, such as aplastic anemia and agranulocytosis, have generally been used as headings in case reports or in studies conducted to determine the incidence and risks of these adverse effects in the population [1,19,20]. Experimental studies to explain the mechanisms are too limited. As a result of examination of bone marrow biopsy samples obtained from agranulocytosis cases, it was suggested that metamizole can stop the maturation of granulocytes to the terminal stage during the Hematopoietic process. The fact that the development of the cells limits at the promyelocyte and myelocyte stage indicates that the effect is in the bone marrow [21]. Studies of an acute promyelocytic leukaemia cell line (HL60) used in granulocyte differentiation assays have shown that metamizole can induce apoptosis and limit differentiation in these cells [5,6]. In another study where Hematopoietic progenitor cells were used directly, the pharmaceutical limited proliferation and formation of CFU-GM colonies starting from a concentration of 1 µM and also caused a significant decrease in the expression of CD66b, a granulocyte marker, at a concentration of 100 µM [7]. In our study, it was found that the proportion of viable cells and the number of CFU-GM colonies decreased starting from 10 µM concentration.

The IC50 value of metamizole on COX and cell lines was studied. It has been reported that IC50



Relative mRNA expression fold-change (Mean±SD)			
Concentrations	1 µM	10 µM	100 µM
OCT4	0.96±0.06	0.71±0.15	0.35±0.09
SOX2	1.01±0.04	0.95±0.05	0.58±0.06
SOX4	1.00±0.04	0.85±0.12	0.47±0.15

Figure 4: A: OCT4, B: SOX4, C: SOX2 relative mRNA expressions at increasing concentrations of metamizole. Kruskal Wallis and Dunn's multiple comparison. The table shows the mean fold change of the groups in the graphs. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001. >2: For OCT4, more than 2-fold reduction vs. all groups. For SOX4, more than 2-fold reduction vs. control and 1 µM groups.

values in COX inhibition vary between 2.55 and 400 µM [22]. However, the values obtained in cell lines are few and vary according to cell type. In a study the values provided after 48 hours of exposure, IC50 values were obtained as HeLa –183.401 µg/mL (~550 µM); HT-29 –316.765 µg/mL (~950 µM); MCF-7 –29.297 µg/mL (~88 µM) [18]. In our study, the IC50 value of metamizole directly for Hematopoietic progenitor cells was obtained for the first time with interesting results. The results were obtained separately from ten samples and the mean value was determined as 92.19±36.31 µM, but the difference between the lowest value and the highest value was more than three times. These results indicate that individual differences in the adverse effects of the pharmaceutical and probably the genetic basis that provides these differences are important. However, this study does not provide results that are based on genetic differences in the effects of the drug, but it does reveal that this needs to be investigated. They also suggest that possible polymorphisms in the COX enzyme may be effective. Perhaps, changes in molecules responsible for Hematopoiesis may also play a role. These changes may be related to transcription factors that carry out the first steps of protein synthesis in many events in cells.

There is too limited knowledge on the effects of NSAIDs on transcription factors in cells. PPARs are transcription factors that play important roles in regulating metabolism, differentiation, development, and tumorigenesis. PPAR activation reduces inflammatory reactions, but in the absence of proinflammatory factors, activation of PPARγ induces COX-2 expression. NSAIDs activate PPARγ as agonists but flufenamic acid, diclofenac, indomethacin and flurbiprofen induce the expression of COX-2 although they directly inhibit this enzyme activity [9]. Another molecule that plays an important role in inflammation is NF-κB. A colorectal cancer model study in rats suggests that NSAIDs inhibit NF-κB and Jak3/Stat3 signaling, downregulating pro-inflammatory cytokines, inflammation to a level that inhibits carcinogenesis [10]. These studies are related to COX mechanisms and inflammation-dependent factors, but indicate that NSAIDs may act on transcription factors. However, drug-induced cytopenia may be related to inhibition of cell growth, development, and especially differentiation. The adverse reactions may develop due to inhibition of transcription factors. Acetylsalicylic acid reduced stem cell proliferation in planarians, which have a very good regeneration ability, and caused delay in blastema growth. Acetylsalicylic acid is thought to reduce mRNA expression of stem cell-related target genes via the STAT3/SOX2/OCT4 signaling pathway [13].

There are studies investigating the roles of OCT4, SOX2 and SOX4 in the proliferation and differentiation of Hematopoietic stem and progenitor cells [23,24,25]. Huang et al. [23] showed that OCT4 activated with OCT4-activating compound 1 (OAC1) increased the expansion of Hematopoietic stem cells derived from cord blood ex vivo by regulating HOXB4 expression and thus the number of cells expressing CD34. HOXB4 (the gene encoding the Hox-B4 homeobox protein) regulates the self-renewal of Hematopoietic stem cells. It was stated in the study that SOX2 and NANOG expressions also increased in these activated cells. Świstowska et al. [24] showed the expression of OCT4 and SOX2 in CD34+ cells isolated from stem cell products obtained after mobilization, similar to our study. They reported that the expression of these transcription factors increased after short-term culture of these cells. Although SOX4 is thought to have a role in lymphocyte development, it was found that it was expressed in early myeloid precursors in mice and the expression continued in proliferating mast cell cultures [25]. An important study revealed that SOX4 is functional in the development of multipotent progenitors of Hematopoietic stem cells. In the deficiency of this transcription factor, development of multipotent progenitors was impaired even though the cells homed in the bone marrow after transplantation [26]. These studies indicate that transcription factors have important roles in the proliferation and differentiation of cells in Hematopoiesis. However, studies to explain the roles of OCT4, SOX2 and SOX4 in Hematopoiesis are too limited. The reductions in the expression seen in our results in metamizole groups suggest that these transcription factors are also active in the control group not exposed to pharmaceutical.

It was determined that the proliferation of PaTu 8988t and Panc-1 (human pancreatic cancer cell lines) was negatively affected by metamizole. The

increase in the number of apoptotic cells after short-term incubation with the pharmaceutical was shown by flow cytometry [16]. It was stated that the pharmaceutical also triggered apoptosis in the HT29 cell line [17]. In addition to these studies, the importance of transcription factors in cancer cells was shown. The expressions of OCT4 and SOX2 were found to be significantly higher in samples obtained from patients with acute lymphocytic leukaemia (ALL) and acute myeloid leukaemia (AML), which are Hematological malignancies, before treatment than after remission. In addition, the expression of the factors was found to be high again during relapse. Therefore, transcription factors are considered as risk factors in cancer [27]. The data suggest that one of the mechanisms in the effect of metamizole on cancer cells may be a decrease in the expression of transcription factors.

Limitation of the study

Detecting the change in differentiation markers by analyzing cell surface proteins of cells via flow cytometry after culture would have provided more data to explain the effect of metamizole. In the design of the study, semi-solid medium containing cytokines was used and it was not possible to grow the cells obtained from the samples produce in a large number of culture dishes. However, this deficiency was compensated to some extent by counting CFU-GM colonies. Studies on OCT4, SOX2 and SOX4 are mostly related to embryonic and pluripotent stem cells. However, it is thought that these transcription factors have important roles in the proliferation and especially differentiation of multipotent cells. Therefore, these transcription factors were preferred in our study. We were able to obtain SOX2 expressions in only 4 samples. This may be due to the fact that the cells we used were directed to differentiation.

Conclusion

In our study, we found that metamizole can reduce the proliferation and differentiation of Hematopoietic stem/progenitor cells by down-regulating the expression of transcription factors. Although the effects show individual differences, the impacts of metamizole on bone marrow-derived cells, especially in cases of agranulocytosis and aplastic anemia, should be investigated in terms of transcription factors. Clinically supported studies to be designed can be evaluated with individual characteristics and can enable the detection of people who are likely to have adverse effects of the pharmaceutical and thus provide safer use of the pharmaceutical in other people. In addition, metamizole is being investigated in terms of its effects in different indications, one of which is its apoptotic effects on cancer cells. The demonstration of possible effects on differentiation and proliferation via transcription factors for these cells may also enable the identification of different indications.

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Ethics Committee Approval: Ethical permission was obtained from the Alanya Alaaddin Keykubat University, Non-Interventional Clinical Research Ethics Committee for this study with date 16.04.2024 and number 2024/08, and Helsinki Declaration rules were followed to conduct this study.

ORCID and Author contribution: The project idea was put forward by **EM (0000-0001-5284-7439)**. The laboratory stages of the study were done by **EM and DNS (0000-0003-2630-6182)**. The findings analysis and writing stages of the study were done by all authors.

Use of artificial intelligence: Artificial intelligence or artificial intelligence-assisted technologies were not used for our article.

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