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Biocompatibility Study of a Cu-Al-Ni Rod Obtained by Continuous Casting

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Abstract: Cu-Al-Nishape memory alloys (SMAs) are widely known for their better properties in comparison to other SMAs (lower production costs, increased ductility, enhanced machinability, reduced liquidus temperature, and decreased hysteresis), but there is still controversy in terms of the biological properties of these materials. The aim of this study was to evaluate biofunctional performances of Cu-Al-Ni alloy produced by continuous casting. The micro-chemical analysis was investigated by SEM/EDX. Immersion tests performed for seven days were used to estimate the quantity of Cu, Al, and Ni ions released in neutral pH and slightly acidic artificial saliva. To assess the biocompatibility of the Cu-Al-Ni alloy samples, MTT assay on fibroblasts and dental pulp cells was performed in indirect and direct contact with samples after 1,3, and 7 days.The study revealed that continuous casting enables the primary fabrication of Cu-Al-Ni rods with a shape memory effect. Samples immersed in artificial saliva with 6.5 pH value showed no significant amounts of released ions, despite the high concentration of copper in the alloy. However, in the acidic environment, the suppression of Cu was 0.14 μg/cm^2, Al 1.9 μg/cm^2, and Ni 0.73 μg/cm^2, and as expected, it was confirmed that Cu-Al-Ni alloy is not corrosion resistant in an acidic environment. In conclusion, this study showed that biocompatibility concerns are related only to materials with a high Cu content in acidic environments. Oppositely, small doses of Cu ions promote cell proliferation, which might be useful in further attempts to enrich different biomedical materials with copper.

Keywords: Cu-Al-Ni rod; microstructure; biocompatibility; Cu ions; cell proliferation; characterisation

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1. Introduction

Advancements in regenerative medicine over the past years have increased research interest in biofunctional materials. Shape memory alloys (SMAs) are the most common intelligent materials and are characterised by a unique correlation between stress, strain, and temperature [1]. Thanks to their unique crystallographic features, such alloys predictably change their shape and composition, adapting to the conditions of the external environment. This distinctive property, known as thermoelastic martensitic transformation, is attractive for biomedical application [2].

Among SMAs, Nitinol has a widespread use in the biomedical field, given its corrosion resistance and remarkable biocompatibility [2,3]. However, the high cost of production of these materials is the reason why, in clinical practice, there is a need for their alternatives.

The production process of Ni-Ti alloy is more complex than Cu-Al-Ni. Cu, Al, and Ni are native metals that can occur in pure and directly usable form [4,5]. Contrary to that, titanium is rare and can be detected only bonded to other metals or as titanium dioxide, known as rutile. The complexity of the extraction process of Ti from rutile (Krolls process) makes this metal less economical than others [6]. Additionally, Ti is highly reactive to

oxidation during the casting process, requiring high vacuum casting furnaces and thus increasing the complexity of Ti alloy production [7,8] as compared to conventional casting procedures. When using a graphite crucible, TiC is usually formed [8], which is undesirable, so even from this point of view, the production of a Ni-Ti alloy has some flaws.

Given the secondary production process of Ni-Ti alloys, its machining is also characterized by severe tool wear, high specific cutting energy, and high strain hardening, which is not the case with Cu-based alloys [7].

Due to abovementioned possible issues during melting and casting of Nitinol, Cu-based alloys appear as a good alternative. These alloys do not require specific manufacturing procedures to ensure the martensitic structure and shape memory effect that are necessary for Nitinol. Cu-based alloys usually have good thermal and electrical conductivity and good thermal stability of the martensitic transformation [9,10]. It is also important to emphasize that Cu-based SMAs are very resistant to degradation resulting from material ageing [10]. Furthermore, they are exceptionally functional materials [11]. Thin ribbons of Cu-Al-Ni SMA have been investigated for possible applications as microsensors and microactuators, i.e., BioMEMS [12]. Furthermore, Cu-Al-Ni alloy has also been studied for potential use in dentistry, for instance for the production of orthodontic archwires [13].

The most widely used Cu-based alloys are Cu-Zn-Al and Cu-Al-Ni alloys [9]. The properties of these ternary alloys are composition-dependent and can be enhanced by adding alloying elements [14]. The addition of nickel has a positive effect on corrosion resistance, while both nickel and aluminium increase mechanical strength. The main issue with the conventional manufacturing of Cu-Zn-Al alloy is zinc's tendency to evaporate during the casting procedure [15] and thus, it is difficult to achieve the desired chemical content of this ternary alloy. From the aspect of melting and casting, Cu-Al-Ni alloy is more stable.

Even though the shape memory effect of Cu-based alloys was established in the early 1960s, up to nowdays, these materials have not been classified as fully biologically compatible, due to their high Cu content. There is not much available data about the application of Cu-Al-Ni alloys in the biomedical field mainly because, by general agreement, Cu is considered to be a toxic element [16]. The main considerations about Cu-containing biomaterials are associated with the risk of acute or chronic copper toxicity [17]. These effects are dependent on the dose, duration, and the route of exposure. Yet, it is also known that Cu is a trace element in the human body, necessary for its proper functioning [18]. Hence, it can be concluded that the influence of Cu on human health may be both positive and negative. Recent studies have suggested its effects on angiogenesis promotion, on bone fracture healing and mesenchymal stem cell osteo-induction, its antineoplastic effects, etc. [13–19]. In addition, Cu demonstrates antibacterial potential and thus can prevent biofilm formation, known as a crucial factor for postoperative infection after implant surgery [20–22].

The fabrication of medical devices can vary, from conventional to 3D computer-guided procedures. Precise control of the process parameters, alloys' chemical composition, and surface integrity are of critical importance in terms of their safe application.

Different methods of fabricating Cu-Al-Ni alloys can result in their different surface characteristics, which is finally reflected in their various functional properties. Contemporary manufacturing techniques are focused on providing the highest quality alloy microstructure after primary production processes. By ensuring the appropriate surface quality of the alloy, the leakage of potentially toxic elements into the tissue can be reduced and, thus, higher safety achieved.

A review of the scientific literature has shown that the optimal chemical composition of Cu-Al-Ni SMAs is about 14 wt.% Al and 3–4.5 wt.% Ni (the remainder represents Cu) [23,24]. Based on this, the selected chemical composition was chosen: 12 wt.% Al, 4 wt.% Ni, and 84 wt.% Cu for our study purposes.

As there are no data related to this specific Cu-Al-Ni alloy, obtained using the continuous casting method, the aims of this present study were to: (1) characterize its microstruc-

ture, (2) determine the ion release from its surface into neutral and acidic pH artificial saliva, and (3) evaluate its biocompatibility in vitro.

2. Materials and Methods

2.1. Production of the Cu-Al-Ni Rod

The Cu-Al-Ni alloy was prepared by vacuum induction melting (VIM) using a Leybold Hereaus furnace (Vantaa, Finland), which could operate up to 60 kW with medium-frequency (4 kHz). For this purpose, the high purity metals Cu, Al, and Ni (99.99 wt.%) were used, delivered by Zlatarna Celje d.o.o. Slovenia. The target alloy composition was 12 wt.% Al, 4 wt.% Ni, and 84 wt.% Cu, and the charge was approximately 10 kg. A clay graphite crucible was used for remelting and melt preparation. Melting was carried out in a vacuum ($p = 10^{-2}$ mbar). Experimental castings were performed with a laboratory scale vertical continuous casting device (Technica Guss, Würzburg, Bayern, Germany) [8,25]. The induction power was 10 kW for the first 10 min, for the next 10 min 20 kW, and in the final 5 min, 30 kW. The casting temperature was in the range 1350–1400 °C. Just before the start of casting, the chamber was filled with argon (purity 5.0). During casting, 25–30 kW was necessary to maintain the casting temperature. For continuous casting, we used a ZrO_2 nozzle stabilised with Y_2O_3 with ϕ = 9 mm and an Fe rod, which allowed the start of pulling the Cu-Al-Ni rod.

Samples' Preparation

After obtaining the Cu-Al-Ni rod, it was cut by electrical discharge machining into discs with thickness of 1.7 mm. A metallographic disc preparation procedure was performed for microstructure examination. The procedure was carried out in such a way that included the hot-mounting of samples and their grinding with abrasive SiC paper in grades of 180–4000 on the grinding/polishing machine BUEHLER Automet 250 and EcoMet 250 (Leinfelden-Echterdingen, Germany). Finally, samples were polished and cleaned with acetone, alcohol, and deionised water in ultrasound. For microstructure observations, the selected samples were etched chemically in an etchant with a chemical composition 5 g $FeCl_3$, 10 mL HNO_3, and 100 mL H_2O; the etching time was 30 s.

To perform the immersion and biocompatibility tests, Cu-Al-Ni discs with polished surfaces were used.

2.2. Chemical Composition Determination and Microstructure Observation

The chemical composition of the Cu-Al-Ni rod was measured with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The spectrometer used was an HP, Agilent 7500 CE, equipped with a collision cell (Santa Clara, CA, USA). The following conditions were used for the ICP-MS: the power was 1.5 kW, Nebuliser—Meinhard, the plasma gas flow was 15 L/min, the nebuliser gas flow was 0.85 L/min, the make-up gas flow was 0.28 L/min, and the reaction gas flow was 4.0 mL/min. The relative measurement uncertainty was estimated as ±3%. Twelve ICP-MS measurements were performed on selected Cu-Al-Ni rod samples.

The microstructure of the Cu-Al-Ni rod was investigated with an optical metallographic microscope, NIKON Epiphot 300 (Tokyo, Japan), with an Olympus DP12 camera (Boston, MA, USA). A Scanning Electron Microscope (SEM), Sirion 400NC (FEI, Hillsboro, OR, USA), with an Energy-Dispersive X-ray (EDX) spectroscope INCA 350 (Oxford Instruments, Abingdon, UK), was used for detailed microstructure observation and the micro-chemical analyses.

The SEM/EDX imaging and analysis were performed at 20 kV accelerating voltage, 1.2 nA probe current, and 7.5 mm working distance with the backscattered electron detector in compositional mode.

2.3. Immersion Testing in Artificial Saliva

Static immersion tests were conducted in a solution of artificial saliva with pH values of 6.5 and 4.5. Saliva composition (pH = 6.5) was 1.5 g/LKCl, 1.5 g/L $NaHCO_3$, 0.5 g/L $NaH_2PO_4H_2O$, 0.5 g/L KSCN, and 0.9 g/L lactic acid [26]. The desired acidity of 4.5 was achieved by adding drops of HCl into the neutral solution of artificial saliva with constant pH control. This low pH value corresponds to the pH of two-day-old biofilm [27]. The Cu-Al-Ni discs were immersed in 5 mL of the test solution in a test tube clogged with a rubber cork. The test duration was 168 h (7 days) at a constant temperature of 37 ± 0.2 °C. Solutions without samples that served as control were also kept for 7 days at 37 °C. Elemental analysis of the resulting immersion solution was performed by the ICP-MS method. For this purpose, the ICP-MS instrument was calibrated with matrix-matched calibration solutions. The purpose of the research was to determine the quantitative release of metal ions (copper, nickel, and aluminium) from Cu-Al-Ni discs immersed in a solution of artificial saliva.

2.4. Biocompatibility Evaluation on Fibroblasts and Dental Pulp Cells

Two types of cells were used in the initial cytotoxicity experiment (MTT): human fibroblasts and dental pulp cells. The corresponding tissues, obtained with written consent from two healthy donors (n = 2), were minced into approximately 1 mm^3 fragments and subjected to the outgrowth method of cell cultivation [28]. The minced tissue was placed in 25 cm^2 culture flasks with the complete growth medium (DMEM/F12 supplemented with 10% FBS and 1% ABAM, all from Gibco, ThermoFisher (Waltham, MA, USA)) and incubated at 37 °C in a humidified 5% CO_2 atmosphere. The cells were passaged regularly upon reaching 80% confluence. The culture medium was changed every 2–3 days. The cells obtained after the 3rd passage were used in the study.

2.4.1. MTT Assay

In accordance with ISO 10993-5:2009, for the indirect test, the Cu-Al-Ni disc was placed in a tube containing 10 mL of CM incubated at 37 °C for 7 days and removed, while the remaining supernatant was used in further experiments. The cells were seeded in a 96-well plate (5.000 cells/well), and the next day 100 µL of supernatant was added to corresponding wells (indirect MTT assay). The ions' content in the medium, originating from the disc, was measured by atomic absorption spectroscopy (AAS).

For the direct MTT test, the discs were placed onto 24-well plates; 20,000 cells/well were seeded onto discs and incubated in freshly prepared growth medium at 37 °C in a humidified 5% CO_2 atmosphere for up to 7 days. The medium was changed every 3rd day. Mitochondrial activity was assessed after 1, 3, and 7 days of treatment.

For the assessment of mitochondrial activity after direct and indirect exposure to the tested Cu-Al-Ni discs, the medium was discarded, and 100 µL of solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, 0.5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated. After 4 h, the supernatant was discarded; dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added to each well; and the plate was placed on a shaker for 20 min, at 250 rpm, in the dark, at 37 °C. The extracted coloured solutions from 12-well plates were transferred into a new 96-well plate. The optical density was measured at 550 nm using a microplate reader RT-2100c (Rayto, Shenzhen, China). Cells without treatment were used as the positive control. The percentage of mitochondrial activity was calculated as the difference fromthe control group.

2.4.2. LDH Assay

The FBs and DPCs were seeded at 5 × 10^3 cells/well in 96-well plates and cultured in the previously mentioned Cu-enriched supernatant. The cells were cultured for 1, 3, and 7 days. After each time point, the amount of lactate dehydrogenase (LDH) was evaluated quantitatively using a colorimetric assay, CyQUANT™ LDH Cytotoxicity Assay (Catalogue no:C20300, ThermoFisher(Waltham, MA, USA)) following the manufacturer's guidelines.

The positive (+) control (total cell death) was generated by adding 10 µL of 10 × X Lysis Buffer to detect the maximum LDH release. The negative (−) control used the medium from the untreated cells at each period. The absorbance was recorded at 560 nm in a microplate reader (RT-2100C Microplate Reader, Rayto, Shenzhen, China).

2.5. Preparation of the Samples for SEM Observations

Preparation of the samples for the SEM observations: after 7 days, the cells were fixed in 2% glutaraldehyde for 2 h at 40 °C, dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 100%), 10 min for each concentration. Residual ethanol was removed with a critical point dryer for 30 min. Samples were gold coated for 120s (thickness 15nm) on a LEICA EMSCD005 (Wetzlar, Germany) machine before Scanning Electron Microscopic evaluation on a JEOL JSM-6610LV (Tokyo, Japan) machine. Secondary electron images of fibroblast cells cultured on the Cu-Al-Ni disc surface were recorded at SEM working parameters of 20 kV voltage, 0.31 nA probe current, and 10 mm working distance.

2.6. Statistical Analysis

The software package GraphPad Prism ver. 9 was used for the analyses (GraphPad Software, Inc., San Diego, CA, USA). Ordinary one-way ANOVA tests and Tukey's multiple comparisons test, with a single pooled variance, were performed in the present study after checking the distribution normality by the Kolmogorov–Smirnov normality test. The values are presented as mean ± SD. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Chemical Composition and Microstructure Observations of the Discs

The measured concentrations of Ni, Al, and Cu in the Cu-Al-Ni rod on the selected discs are shown in Table 1. The obtained results are adequate and do not deviate significantly from the nominal chemical composition.

Table 1. ICP-MS chemical composition of Cu-Al-Ni rod (in wt.%).

Al	Ni	Cu
12.0	3.9	rest
11.9	4.0	rest
12.0	3.9	rest
12.1	3.9	rest
11.9	4.0	rest
12.0	4.0	rest
	Mean	
11.98	3.95	84.07
	Std. Deviation	
0.07	0.05	0.05

Figure 1a,b represent the optical microstructure of the Cu-Al-Ni rod in the cross-sectional areas. Microstructural examination showed that the primary grains were oriented preferably to the centre of the rod, which is consistent with their growth from the outer surface of the rod towards its interior—just as solidification took place during the process of the continuous casting of the rod. The primary grains are in the size range of 100 µm. Martensitic needles are visible, and they extend over the primary grains. The microstructure thus consists of two phases and is quite inhomogeneous. Moreover, the microstructure seems fully martensitic.

A more detailed EDX analysis showed no significant difference in the chemical composition of the two phases (Table 2). An example of such a two-phase SEM microstructure and the performance of EDX point analysis is presented in Figure 2: spectrums 1 and 2 for martensitic needle, spectrums 3 and 4 for primary grains. The obtained results of ICP-MS and EDX analysis are very consistent, and deviations are minimal.

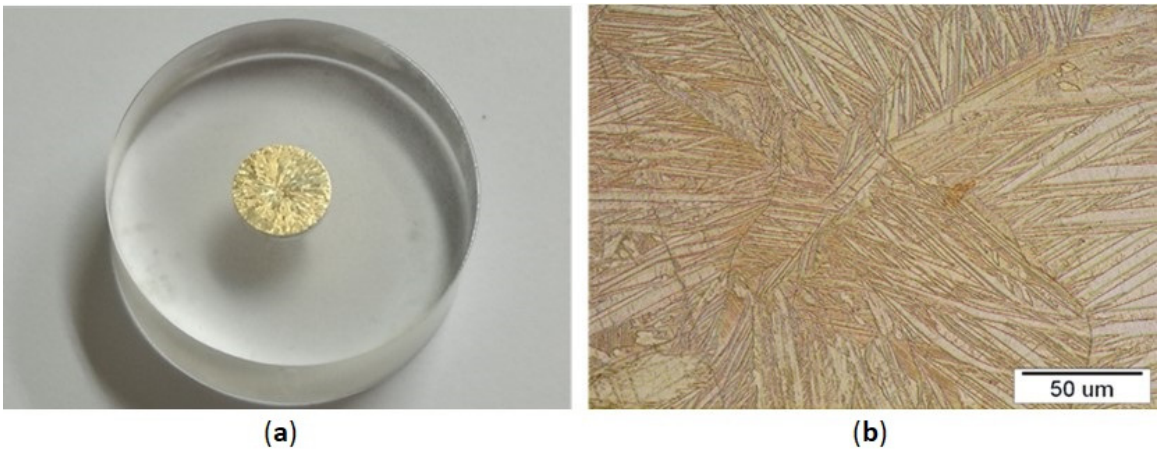

Figure 1. (**a**) Macro view of a Cu-Al-Ni disc; (**b**) etched optical microstructure.

Table 2. EDX chemical composition of the phases in the Cu-Al-Ni rod (in wt.%).

Spectrum	In Stats.	Al	Ni	Cu	Total
Spectrum 1	Yes	12.06	2.94	85.00	100.00
Spectrum 2	Yes	11.25	4.04	84.71	100.00
Spectrum 3	Yes	13.16	3.37	83.47	100.00
Spectrum 4	Yes	11.56	3.96	84.48	100.00
Mean		12.01	3.58	84.42	100.00
Std. Deviation		0.84	0.52	0.67	
Max.		13.16	4.04	85.00	
Min.		11.25	2.94	83.47	

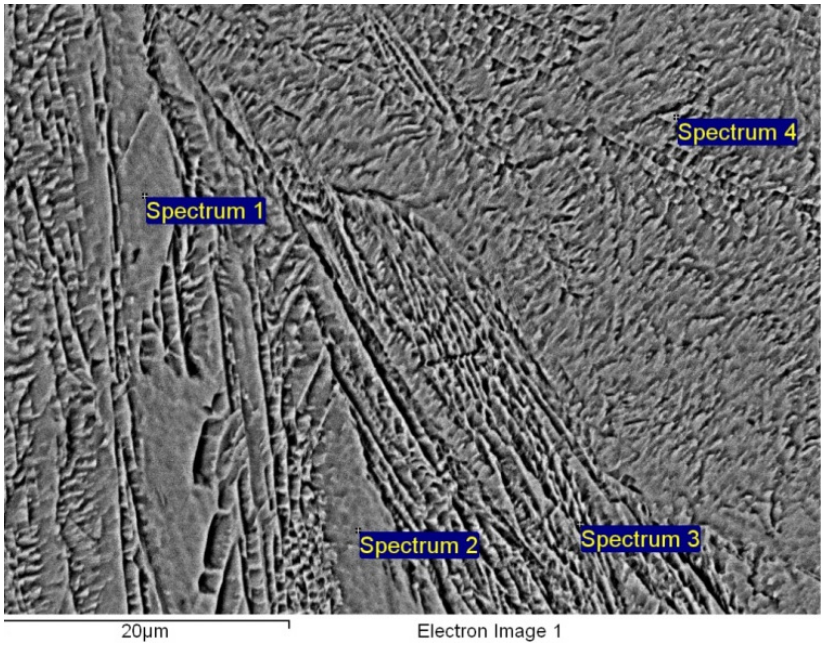

Figure 2. SEM characteristic microstructure of the Cu-Al-Ni disc with presentation of the EDX analysis sites.

3.2. Immersion Testing in Artificial Saliva

The quantities of Al, Ni, and Cu ions released in the immersion testing per surface unit during seven days are shown in Table 3 (measured with ICP-MS). Immersion in a solution of artificial saliva with pH 6.5 after 7 days did not induce significant ion release, nor are these amounts clinically significant. Al and Ni ions' release was 0.02 and Cu was

0.11 μg/cm^2. The acidity of the solution, expectedly, increased the release of metal ions, and significantly different concentrations of Ni and Al ions were detected in the solutions. Al ion release was 95 times higher and Ni ion release was 36 times higher in acidic pH compared to neutral pH. Only a slight increase of Cu ions release was noticed in the acid pH (0.14 μg/cm^2) compared to the neutral pH medium (0.11 μg/cm^2).

Table 3. Al, Ni, and Cu ions' concentrations in artificial saliva pH = 6.5 and pH 4.5 after 7 days of immersion (values are given in μg/cm^2).

	pH = 6.5			pH = 4.5		
	Al	**Cu**	**Ni**	**Al**	**Cu**	**Ni**
Blank solution	0.06	0.08	<0.01	0.02	0.17	<0.01
Cu-Al-Ni disc	0.02	0.11	0.02	1.9	0.14	0.73

3.3. Biocompatibility Results

3.3.1. MTT Assay

The content of Cu ions released in the growth medium, measured by AAS, was 6.33 μg Cu/mL. The content of Al and Ni ions was below the detection limit. Indirect exposure of fibroblasts to the ions-containing supernatant led to increased mitochondrial activity in comparison to the control (mean = 100%). After 24 h of exposure, cell proliferation had a mean value of 113%; after the third day it reached a mean value of 191%, and after the seventh day the mean value was 367%—Figure 3a. There was a statistically significant difference between all groups.

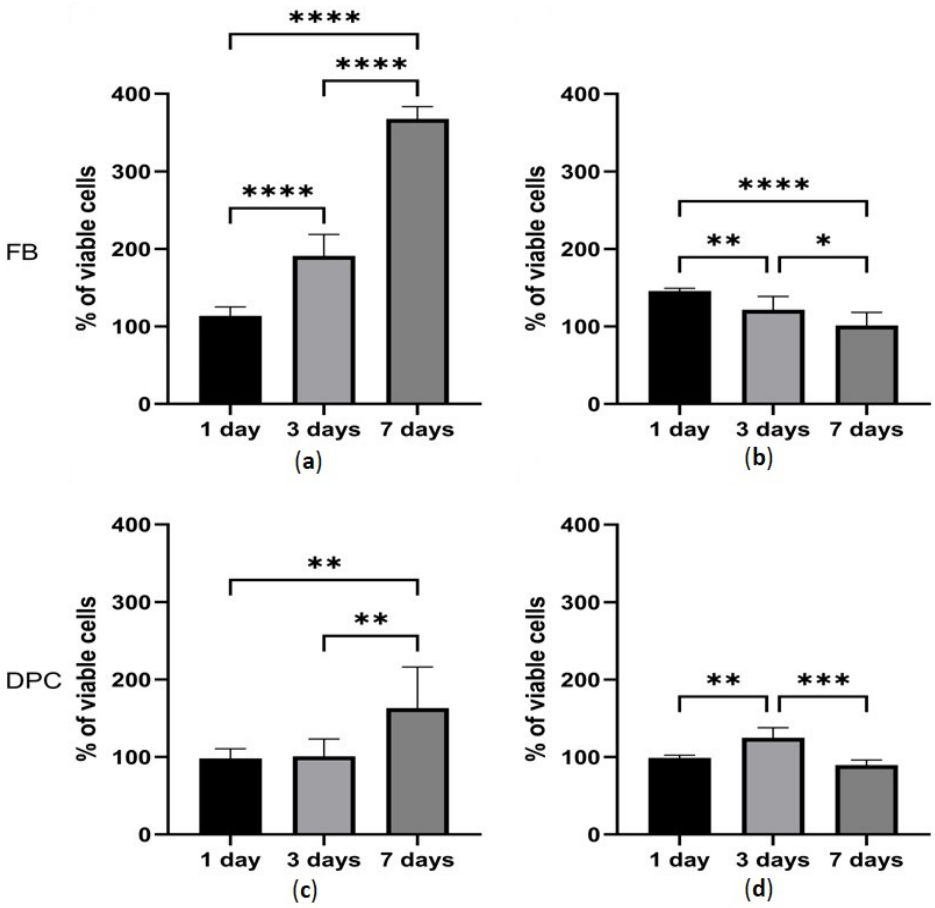

Figure 3. MTT assay. Cell viability for fibroblasts (FB) and dental pulp cells (DPC): (**a**) indirect treatment of FB; (**b**) direct contact of FB with the Cu-Al-Ni disc; (**c**) indirect treatment on DPS; (**d**) direct contact DPC with the Cu-Al-Ni disc, measured after the 1st, 3rd and 7th days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Direct exposure of fibroblasts to the tested Cu-Al-Ni discs led to increased cell proliferation after the firstday (mean = 145%) and then to a decrease after the third(mean = 121%) and seventhdays (mean = 101%) (Figure 3b).

Similar behaviour was observed in the dental pulp cells. The results of the indirect MTT assay showed an increase in cell proliferation of the tested Cu-Al-Ni discs in contact with Cu-enriched supernatants between the first(mean = 98%), third(mean = 100%) and seventhdays (mean = 163%) (Figure 3c).

Contrary to that, the mitochondrial activity of cells grown directly on the tested Cu-Al-Ni discs showed a proliferation tendency only during the observation time between the first(mean = 98%) and thirddays (mean = 124%). After 7 days of direct exposure, there was a decrease in mitochondrial activity (mean = 89%) (Figure 3d).

Overall, no cytotoxic effect could be established, neither during indirect nor direct exposure of cells to the Cu-Al-Ni disc, while indirect exposure led to a linear stimulation of proliferation that was more pronounced in the fibroblasts than in the dental pulp cells.

3.3.2. LDH Assay

The LDH assay confirmed the results of the MTT, i.e., the examined Cu-Al-Ni disc did not elicit cytotoxicity against FBs and DPCs after indirect treatment with the Cu-enriched medium (Figure 4). A significantly lower level of LDH activity was noted in the cells after one, three, and seven days of indirect treatment ($p < 0.05$), compared to the positive control(total cell death). Indeed, the cells' LDH activity was similar to the negative control (untreated cells). There were no differences in LDH activity between the FBs and DPCs.

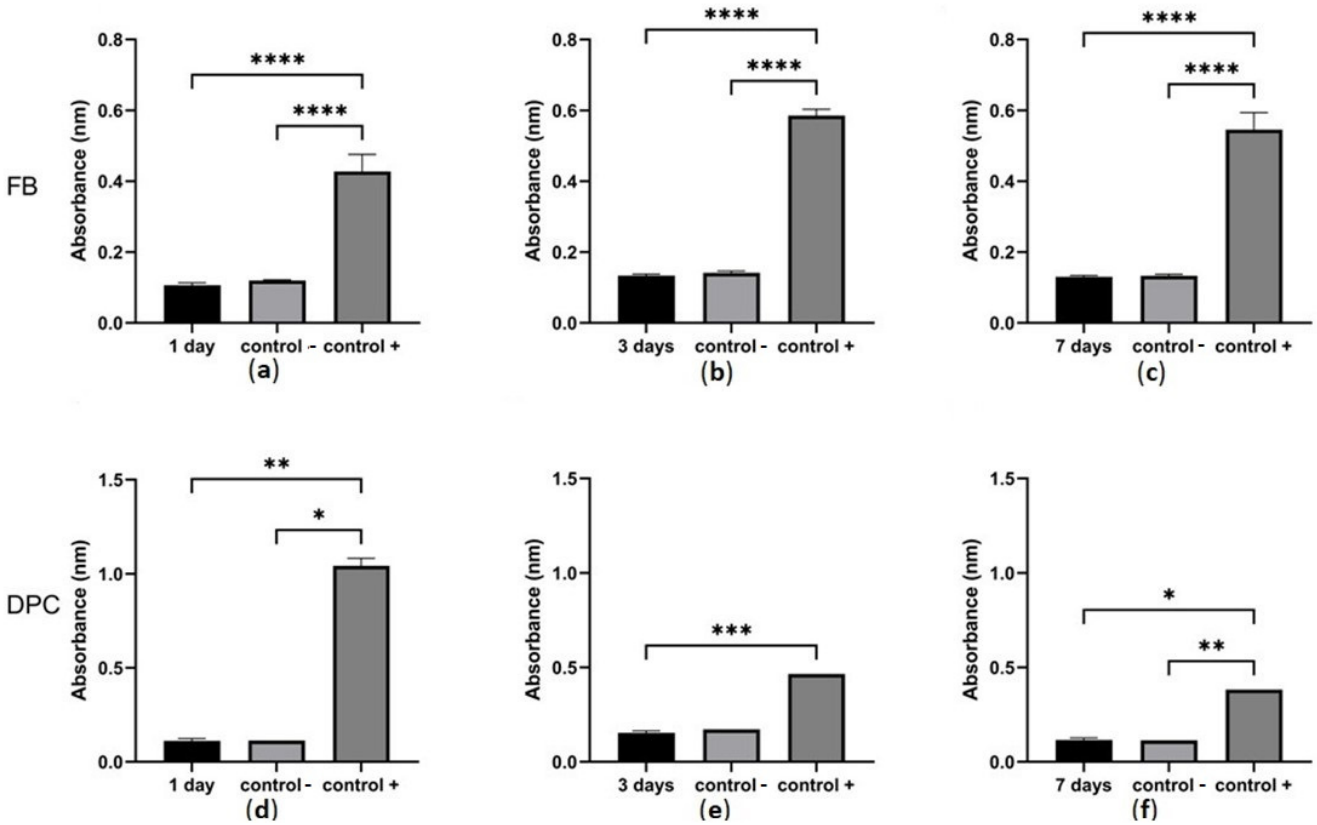

Figure 4. LDH activity measured at the (**a**) 1st, (**b**) 3rd, and (**c**) 7th days of indirect treatment of fibroblasts (FB) and the (**d**) 1st, (**e**) 3rd, and (**f**) 7th days of indirect treatment of dental pulp cells (DPC) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Scanning Electron Microscopy

Evaluation of the cell growth on the Cu-Al-Ni discs' surface determined a good attachment of fibroblasts onto the disc surface—as shown in Figure 5a,b.

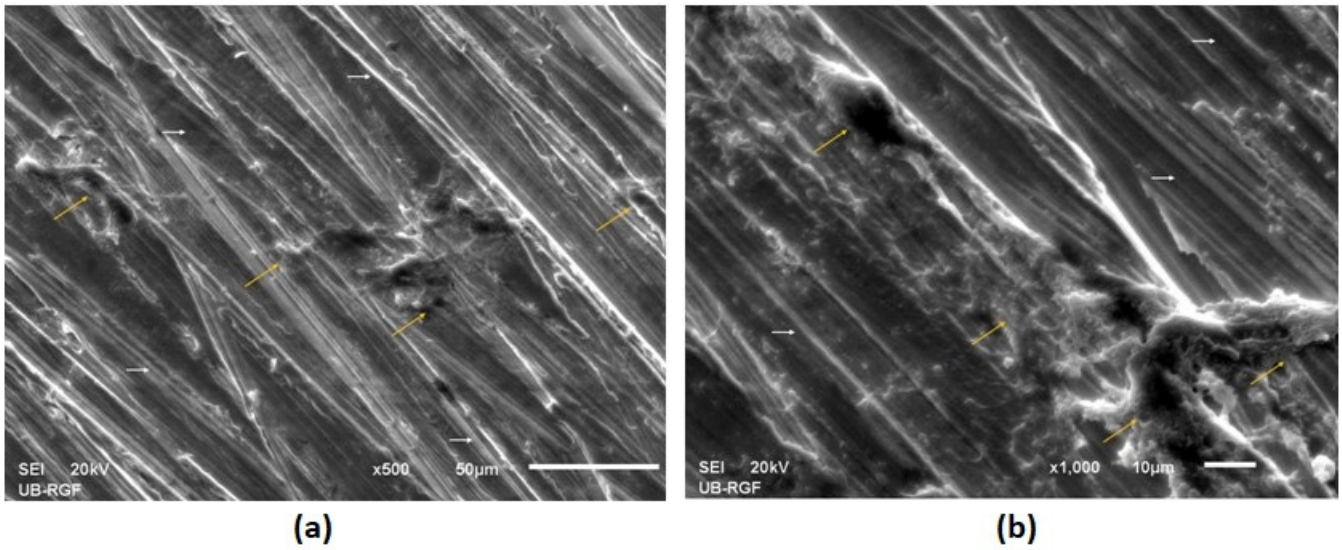

Figure 5. SEM micrograph: (**a**) 500× magnification; (**b**) 1000× magnification of fibroblast cells cultured on the Cu-Al-Ni disc surface. White arrows indicate material relief, and yellow arrows indicate cells.

4. Discussion

This study demonstrated the biocompatibility of a continuously cast Cu-Al-Ni rod investigated as a novel material for potential usage in medicine. Samples' microstructures confirmed the occurrence of martensite, indicating that continuous casting enables the primary fabrication of Cu-Al-Ni rods with a shape memory effect. However, it seems that the microstructure of the cast Cu-Al-Ni rod may not be a sufficient argument to consider this type of alloy for possible biomedical purposes. The main reason for scepticism is the Cu-Al-Ni disc's poor corrosion resistance in an acidic environment. Yet, discs immersed in artificial saliva with a 6.5 pH value did not show significant amounts of released ions. The high concentration of Cu in the disc did not result in proportionally high Cu ion release. It is known that the good surface quality of a disc acts like an ion diffusion barrier, but when the solution's pH value decreased, the release of metal ions increased. A similar behaviour has been observed in previous studies [29–31]. As expected, the results of this study confirmed that Cu-Al-Ni discs are not corrosion resistant in an acidic environment. Nevertheless, the release of Cu ions in the acidic medium, in comparison to Ni and Al ions, was relatively limited, indicating that the chemical composition of the medium is an important variable for the corrosive behaviour of such a ternary alloy.

The low levels of Cu ions released in the solution could be explained by the composition of saliva. It is known that atoms such as nitrogen, sulphur, and phosphorus present in the organic compound are classified as corrosion inhibitors. These atoms have a high affinity for Cu and thus support the production and formation of protective films on the copper surface [14,15]. Even though Cu ions were not released at high doses, Ni ions' release was over the tolerable upper level for humans. The maximum allowable dose of Ni ions for humans is 0.5 $\mu g/cm^2$ per week [32]. As far as Al ions are concerned, it is known that trivalent Al is a highly reactive metal. Immersed in a pH-neutral medium, it forms aluminium oxide, hydroxide, etc. These compounds act as protective surface oxide layers preventing further corrosion. However, Al compounds are soluble in acidic environments. Contrary to nickel, it has not been reported that metallic Al, its oxides, and Al salts exert genotoxic or carcinogenic effects, even though these metal compounds can promote negative effects on the central nervous system, bone tissue, and the liver [33]. The daily aluminium intake from food is 4000–9000 micrograms, not to mention other sources, such as drinking water, deodorants, air, medications, etc. [33].

The findings of our study could lead to further investigations in the context of secondary surface treatments and alloying procedures. To improve the corrosion resistance of Cu-Al-Ni rod, surface protection procedures such as Atomic layer deposition can also be used [34]. This method prevents the release of ions from metallic materials' surfaces. It was also reported that by alloying Cu with Cr, Mo, Ti, etc., corrosion resistance in an acidic environment was improved [35–37].

Given that biological functions of copper in human cells define the possibilities of its application in the future, our research demonstrated the lack of Cu cytotoxic effect on two types of cells (gingival fibroblasts and dental pulp cells) grown directly on the Cu-Al-Ni disc's surface and in contact with the ion-enriched supernatants (obtained by soaking the discs into culture medium). Fibroblasts from human gingival mucosa were used, in the first place, because they show a high sensitivity in standard cytotoxicity tests but also because copper could potentially find applications in different fields of dentistry and thus come into contact with FBs. Namely, in recent years, several studies have pointed to the beneficial effect of the addition of copper to restorative dentistry materials. For instance, a graphene oxide-copper nanocomposite promoted the proliferation and adhesion of DPCs [38].

In order to confirm the findings on fibroblasts' cultures, the same biocompatibility tests were applied on dental pulp cells, and rather similar results were obtained, i.e., the Cu ions in the culture medium enhanced dental pulp cell proliferation.

Considering the fact that Ni and Al were not detected in the conditioning medium for indirect biocompatibility, the experimental design consisted of the evaluation of copper ions on cellular behaviour. Moreover, these results were expected, given the previously obtained results of the static immersion test in pH-neutral artificial saliva. However, if we consider studies based on cytotoxic effect of Cu, Al, and Ni elements, the results showed that concentrations of Cu lower than 8 μg/mL and Ni lower than 3 μg/mL are not cytotoxic for the L929 cells (fibroblast cell line) [39]. Furthermore, it has been reported that Al is toxic, primarily for nervous and osseous tissue, at concentrations between 0.016–0.225 μg/mL [40].

The LDH assay corroborated the lack of cytotoxicity of the examined alloy on fibroblasts and dental pulp cells. It is well known that the biosafety of Cu-containing alloys is highly dependent on the dose, duration, and the route of exposure [12]. With this in mind, studies have addressed the effect of different concentrations of copper on the cellular response [41,42]. There is no consensus regarding the amount of copper in alloys that would enhance/improve biological responses, and only a few studies have addressed this issue. Liu et al. have found that a concentration of copper over 7.5 μg/mL leads to some degree of cell apoptosis [43]. It was documented that a concentration of copper sulphate of 62.5 μg/mL produces 70% of cell viability, and 220 μg/mL induces 50% lethality in human liver carcinoma cells [44]. It may be difficult to compare the results because different cells or cell lines have been used, but there is a general agreement that cell viability decreases as the concentration of copper increases [45].

The present study focused on a single type of alloy with a specific chemical composition, and this fact represents a limitation. Future research should consider alloys with different Cu contents in order to precisely establish which Cu levels are biocompatible (or cytotoxic) for a given type of cells.

5. Conclusions

Within the performed research, we came to the following findings:

1. Continuous casting enables the primary fabrication of Cu-Al-Ni rods with a fully martensitic structure.
2. The behaviour of the Cu-Al-Ni alloy obtained by continuous casting, when in pH-neutral artificial saliva, did not result in significant metal ion release. On the other hand, poor corrosion resistance in the acidic environment remains a concern, as thesolution's acidity increases the release of Al and Ni ions. For further investigations in the

context of better corrosion resistance in an acidic environment, surface modifications and/or different alloying procedures should be used.

3. Taking into consideration the all-encompassing results of the biocompatibility tests, it was shown that Cu at low concentrations had a strong stimulatory effect on cell proliferation, especially on fibroblasts. After 7 days of exposure to 6.33 μg Cu/mL, cell proliferation increased to 367%.
4. The LDH assay confirmed the results of the MTT, i.e., the examined Cu-Al-Ni disc did not elicit cytotoxicity against FBs and DPCs after indirect treatment with the Cu-enriched medium.

The findings of this study might be useful in further attempts to enrich different biomedical materials with copper in order to induce tissue repair. They could also be used for metallic materials' coating strategies to promote osseointegration. In other words, there is much room for future investigations of copper-enriched biomaterials, especially for application in oral and maxillofacial surgery and regenerative endodontics.

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Abbreviations

AAS—Atomic absorption spectroscopy, ABAM—Antibiotic-antimycotic, ANOVA—Analysis of variance, CM—Complete growth medium, DMEM–Dulbecco's Modified Eagle's Medium, DPCs—Dental pulp cells, EDX—Energy Dispersive X-Ray Analysis, FB—Fibroblasts, FBS—Foetal Bovine Serum, ICP-MS—Inductively Coupled Plasma-Mass Spectrometry, LDH—lactate dehydrogenase, MTT—Colorimetric assay for measuring cell metabolic activity, Nitinol–Commercial name for an equiatomic Ni-Ti alloy, VIM—Vacuum induction melting, SEM—Scanning Electron Microscope, SMAs—Shape Memory Alloys.

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