In vivo cytotoxic evaluation of Ti–Ni–Fe shape memory alloys

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A series of Ti based shape memory alloys with composition of $Ti_{50}Ni_{48}Fe_2$, $Ti_{50}Ni_{47}Fe_3$ and $Ti_{50}Ni_{45}Fe_5$ were developed by vacuum arc melting under a purified argon atmosphere. The study was designed to evaluate *in vivo* cytotoxicity of the Ti–Ni–Fe shape memory alloy system. The materials were implanted in rabbits, and blood examination and histology of various vital organs (liver, heart and kidney) were performed to determine cytotoxicity of these alloy systems, if any, after 4, 8 and 12 weeks. The results showed that Ti–Ni–Fe alloy neither was cytotoxic nor has any systemic reaction on living system in any of the test performed. Implantation shows good compatibility and a potential of being used directly in *in vivo* system.

Introduction

Ti based shape memory alloys (SMAs) are extensively used as fascinating biomaterials due to their better corrosion resistance, biocompatibility, better mechanical and damping properties, unique shape memory effect and pseudoelasticity. These materials must therefore be able to ensure the functional requirements (e.g. strength, fatigue strength, rigidity and electrical conductivity) along with their ability to continue performing for a long period of time without deterioration of the material itself or undesirable effects induced in biological environment.[1] The term 'biocompatibility' encompasses many different properties of the materials. However, two most important aspects of the biomaterial screening refer to evaluation of cytotoxic effects on nearby and remote organ using histological methods, and their blood compatibility behaviour was assessed using blood profiling.[2] Moreover, concerns exist regarding their use in certain biomedical scenarios due to the known toxicity of Ni and conflicting reports of Ni–Ti corrosion resistance, particularly under dynamic loading.[3] *In vitro* toxicity tests clearly have an important role to play in providing appropriate data for the safety assessment of materials that are being implanted in biological system.[4] However, the non-zero covalent nickel ions, having toxic effects, might release from the alloy in the body fluid environment because of biocorrosion or erosion.[5]

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Titanium alloy biocompatibility depends very much upon the physicochemical properties of their oxide layers, which are in direct contact with the tissues. The oxide films on titanium bioalloys contain dense and stable TiO_2, which show osseointegration ability. Like pure Ti, Ni–Ti surfaces are composed primarily of passive TiO_2 layer, which renders the alloy bioinert characteristics and resistant to corrosion.[3,6]

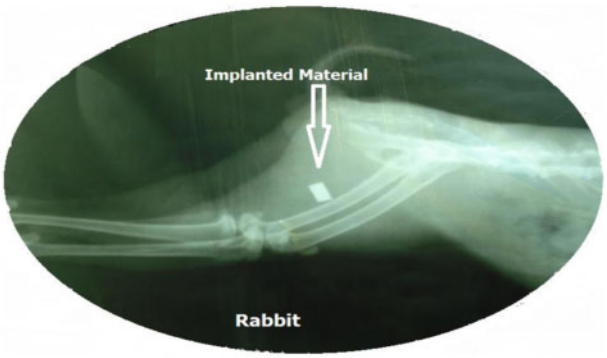
Cytotoxicity evaluation has been performed for the specimens of pure metals, alloys and metal salts. It is also proven that the results of the cytotoxicity evaluation correlate well with the levels of inflammation observed by *in vivo* implantation tests.[7] Previous cytological experiment of titanium alloys had shown neither toxic effects on human fibroblasts nor any abnormal findings such as inflammation in the tissues surrounding titanium implants in humans or in animals.[8]

Despite the potential benefits of Ti–Ni–Fe SMAs, their development for biomedical applications is still in infancy. A systematic study of the electrochemical behaviour of Ti–Ni–Fe system was reported earlier,[9] but till now, no extensive study has been performed to assess the biocompatibility of Ti–Ni–Fe SMAs. Therefore, the study was designed to evaluate cytotoxic effects of $Ti_{50}Ni_{48}Fe_2$, $Ti_{50}Ni_{47}Fe_3$ and $Ti_{50}Ni_{45}Fe_5$ SMAs on far tissue/organ, and their biochemical behaviour was assessed by evaluating different blood parameters *in vivo*.

Experimental

Implanted materials

Three Ti–Ni–Fe button shaped alloy compositions, i.e. TNF1 ($Ti_{50}Ni_{48}Fe_2$), TNF2 ($Ti_{50}Ni_{47}Fe_3$) and TNF3 ($Ti_{50}Ni_{45}Fe_5$), were prepared by arc melting the high purity constituents under argon atmosphere. The carefully weighed and cleaned base metals were melted in an arc furnace under high purity argon environment. Titanium getter was used in the furnace to remove



1 Radiograph 12 weeks after operation showing location of Ti–Ni–Fe implant in leg of rabbit

traces of oxygen in argon. Each alloy was remelted five times to ensure homogeneity. The mass loss after six melting cycles was negligible; therefore, the actual composition was considered unchanged. The as cast samples were homogenised in a tube furnace at 1000°C for 2 h in argon atmosphere followed by quenching in iced water. The heat treated specimens were hot rolled at 850°C (in austenite condition) to an ultimate reduction of 90%. After the hot rolling, the specimens were cut into $5 \times 5 \times 1$ mm strips.

Subject animals and surgical procedure

Twelve sexually mature male rabbits, age ≥ 3 years and weighing from 2·6 to 3·3 kg, were used as subject animals. The animals were housed in animal laboratory having a thermostatically controlled room at 37°C with a relative humidity of $50 \pm 10\%$. The room was artificially illuminated with 12 h of light and 12 h of darkness. Subject animals were fed a standard pelleted diet. The animal tests were performed after compliance of animal welfare rights with approval by the Animal Welfare Society of Bahauddin Zakariya University, Multan, Pakistan. Twelve male rabbits, divided in three groups, were used throughout the study. In each group, three out of four enrolled rabbits received a Ti–Ni–Fe alloy implant subcutaneously for 4, 8 and 12 weeks, while the fourth animal acted as an internal control. All animals were slaughtered using an overdose of diethyl ether as volatile anaesthetic after completion of the above mentioned test duration. Standard plain radiographs of the animals were taken to check the implant position.

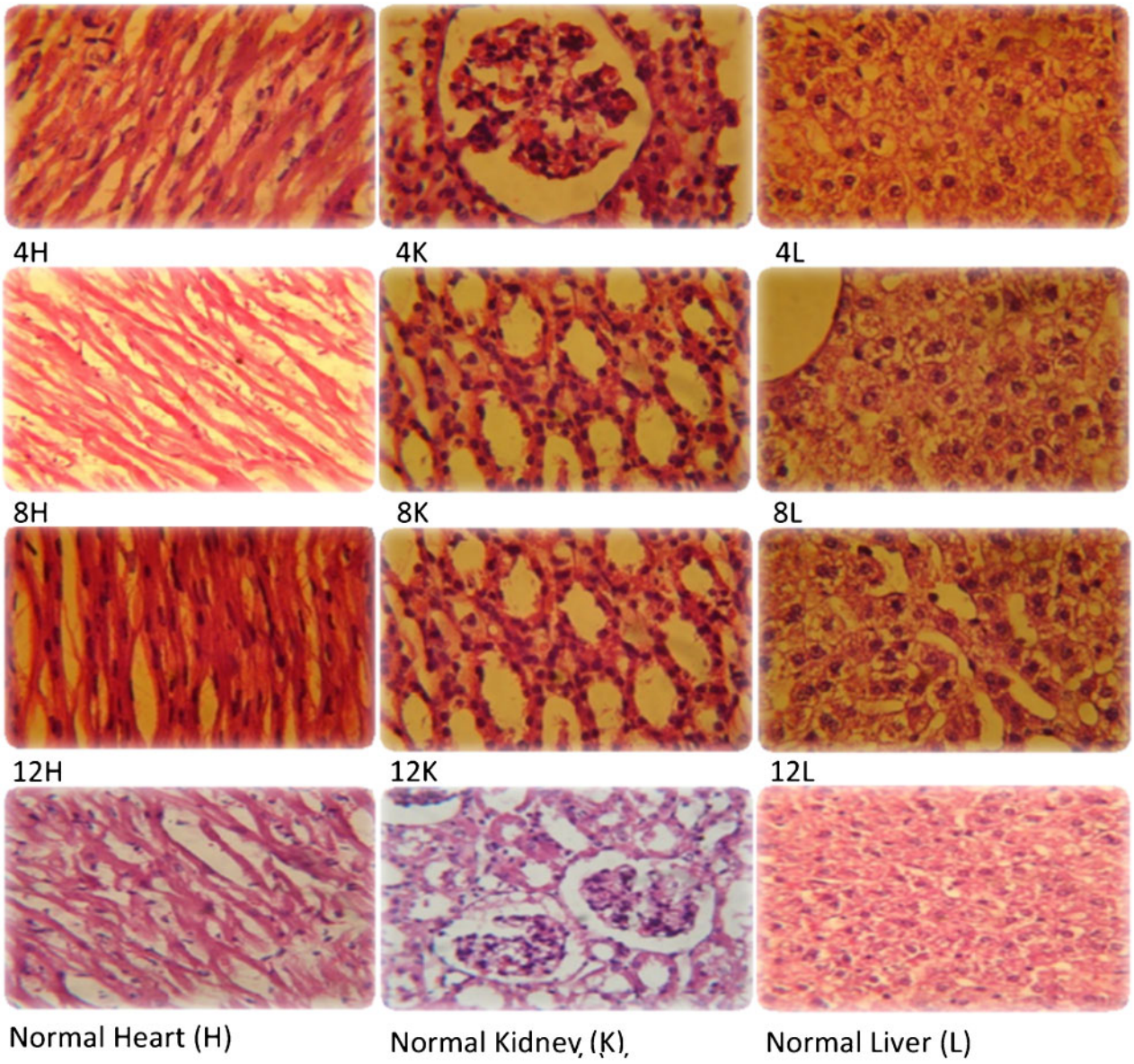
Metallic implant strips were soaked in 20% (v/v) HNO_3 for 20 min, washed (ASTM F86-12)[10] and finally sterilised by autoclaving. Following the anaesthesia, incisions were made under aseptic conditions, after which the TNF1, TNF2 and TNF3 metallic strips were implanted subcutaneously at the lateral aspect of right hind limb. Sample migration was avoided by closing the fascia with a non-absorbable suture point. Radiograph of every implanted rabbit was recorded every 4, 8 and 12 weeks to check the status of the prostheses (Fig. 1).

Blood sample collection

Blood sample from implanted rabbits was taken after 4, 8 and 12 weeks in order to evaluate haemoglobin, total erythrocyte count, packed cell volume, erythrocyte sedimentation rate, total platelet count, total leukocyte count, neutrophils, lymphocytes and monocytes in blood over time. Blood samples were collected from jugular vein of the rabbits using disposable syringe with 23 gauge stainless steel needle. Blood samples were transferred to blood collection tubes conditioned with lithium heparin and stored at refrigerated temperature,

Table 1 Statistical analysis of normal, TNF1, TNF2 and TNF3 implants and blood examination after implantation in soft tissues of rabbits for 4, 8 and 12 weeks

Parameters	Time period/ weeks	*F* test	Normal ($n=6$)	TNF1	TNF2	TNF3	Significance
Haemoglobin/g dL^{-1}	4	3·12	12·000 ± 0·62	12·800 ± 0·50	14·000 ± 0·53	13·000 ± 0·91	NS
	8	3·79	12·500 ± 0·47	13·200 ± 0·41	13·800 ± 0·69	12·800 ± 0·61	NS
	12	3·24	12·200 ± 0·91	13·100 ± 0·41	13·600 ± 0·71	12·700 ± 0·35	NS
Red blood cells/10^6 μL^{-1}	4	3·53	7·0667 ± 0·5	6·8000 ± 0·5	6·4000 ± 0·5	5·8000 ± 0·61	NS
	8	3·15	7·2000 ± 0·56	6·4000 ± 0·5	6·3000 ± 0·55	6·0000 ± 0·43	NS
	12	3·35	7·6000 ± 0·73	6·8000 ± 0·5	6·5000 ± 0·38	6·2000 ± 0·59	NS
Packed cell volume/%	4	3·00	42·000 ± 0·71	38·000 ± 0·46	36·000 ± 0·61	36·000 ± 0·81	NS
	8	3·05	44·000 ± 1·12	34·000 ± 0·93	34·000 ± 0·51	38·000 ± 0·72	NS
	12	3·99	46·000 ± 0·40	36·000 ± 0·81	38·000 ± 1·18	40·000 ± 0·46	NS
Erythrocyte sedimentation rate/mm h^{-1}	4	3·67	6·0000 ± 0·42	4·0000 ± 0·47	5·0000 ± 0·162	5·0000 ± 0·48	NS
	8	3·99	3·0000 ± 0·42	4·0000 ± 0·26	2·0000 ± 0·46	3·0000 ± 0·46	NS
	12	3·09	4·0000 ± 0·61	5·0000 ± 0·19	3·0000 ± 0·42	5·0000 ± 0·46	NS
Platelets/10^5 μL^{-1}	4	3·43	4·5000 ± 0·67	5·0000 ± 1·16	4·0000 ± 0·47	5·4000 ± 0·41	NS
	8	3·28	4·8000 ± 0·41	5·2000 ± 0·45	4·2000 ± 0·91	5·6000 ± 0·75	NS
	12	3·76	4·4000 ± 0·83	4·8000 ± 0·71	4·4000 ± 0·49	5·2000 ± 0·481	NS
Total leukocyte count/10^3 μL^{-1}	4	3·07	7·0000 ± 0·71	7·2000 ± 0·64	8·4000 ± 0·62	8·6000 ± 0·85	NS
	8	3·16	7·2000 ± 0·46	7·6000 ± 0·71	8·6000 ± 0·62	8·8000 ± 1·42	NS
	12	3·77	6·8000 ± 0·28	7·2000 ± 0·41	8·0000 ± 0·71	9·0000 ± 1·82	NS
Neutrophils/%	4	3·89	55·000 ± 0·81	60·000 ± 0·41	54·000 ± 0·41	45·000 ± 1·03	NS
	8	3·47	45·000 ± 0·96	65·000 ± 0·48	57·000 ± 1·01	52·000 ± 0·48	NS
	12	3·20	40·000 ± 1·24	58·000 ± 0·46	53·333 ± 1·09	48·000 ± 0·83	NS
Lymphocytes/%	4	3·62	44·000 ± 1·15	39·000 ± 0·44	45·000 ± 1·08	54·000 ± 0·72	NS
	8	3·78	54·000 ± 0·41	32·000 ± 0·51	42·000 ± 0·47	47·000 ± 0·91	NS
	12	3·09	59·000 ± 0·91	40·000 ± 0·61	46·000 ± 0·67	51·000 ± 1·92	NS
Monocytes/%	4	3·98	1·0000 ± 1·43	1·0000 ± 0·58	1·0000 ± 0·81	1·0000 ± 0·71	NS
	8	3·00	1·0000 ± 1·09	3·0000 ± 0·87	1·0000 ± 0·91	1·0000 ± 0·49	NS
	12	3·01	3·0000 ± 0·51	3·0000 ± 0·81	3·0000 ± 0·68	3·0000 ± 0·41	NS



2 ***In vivo*** **tissue response to alloy designated as TNF1 in rabbits of three groups showing vital organ histopathology [liver (L), kidney (K) and heart (H) at fourth, eighth and twelfth weeks post-implantation of TNF1 alloy]**

i.e. 4°C, until analysis. The animals were then slaughtered using diethyl ether as volatile anaesthetic.

Histology of soft tissue

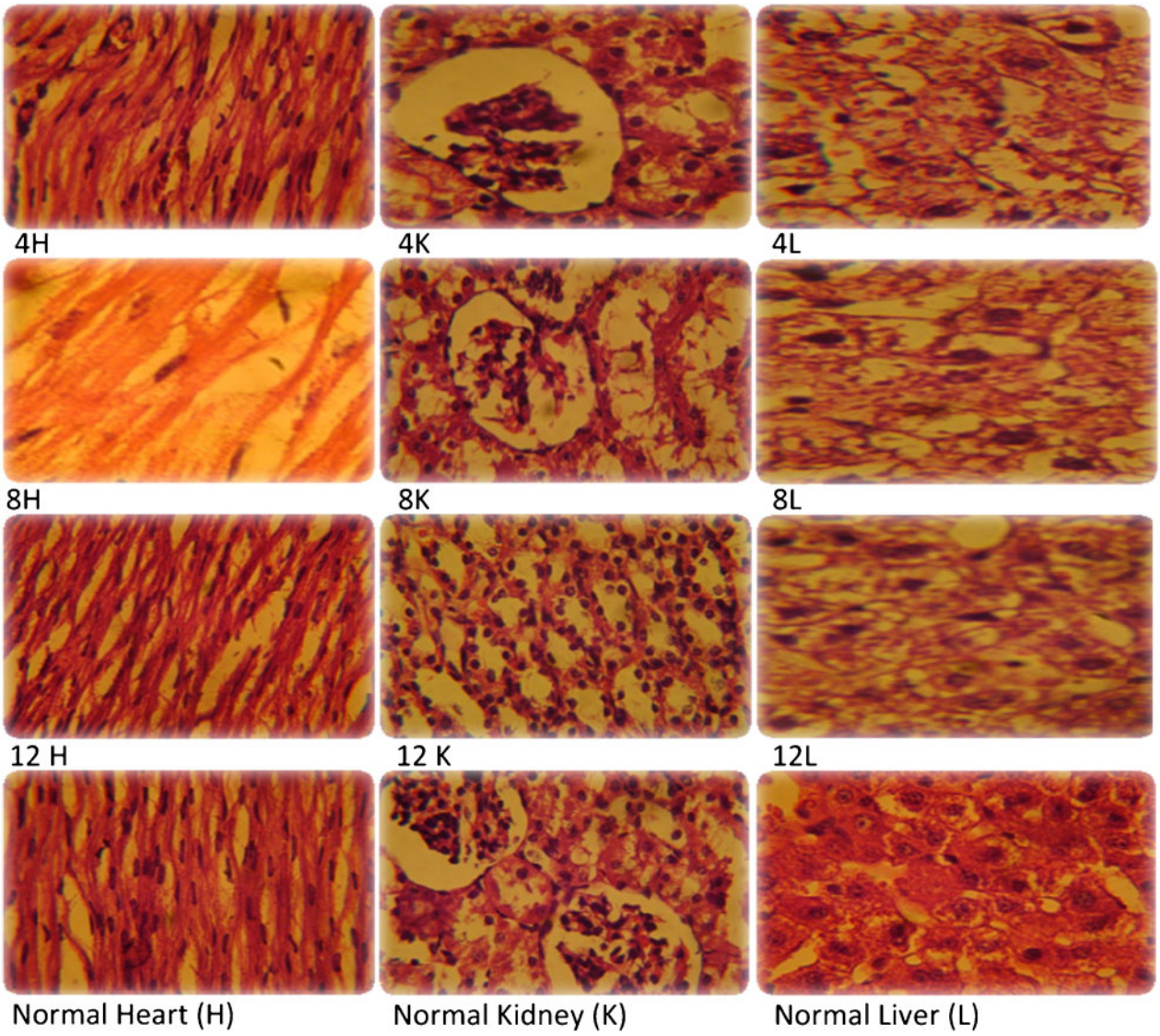
Tissue samples from three vital organs including heart, liver and kidney were collected from rabbits after slaughtering and immediately fixed in 10% neutral buffered formalin. After removal of formalin during overnight tap water washing, tissue blocks were transferred through a series of alcohol water solution as follows: 70% ethanol, 80% ethanol, 90% ethanol and absolute ethanol. Tissues were processed for histological examination by paraffin embedding method. Section thickness was maintained by cutting at 4 μm, which was stained with haematoxylin and eosin[11] for visualisation under a microscope.

Statistical analysis

Statistical analysis was performed to check the mean of all blood parameters in weeks if equal or not, one way ANOVA using MINITAB followed by z test, which determined the mean values. The standard level of significance was kept at $\alpha=0{\cdot}05$ and compared with p value. Values are expressed as mean $\pm$ standard deviation.

Results and discussion

Twelve rabbits divided in three groups were implanted with TNF1, TNF2 and TNF3 SMAs for different time periods (4, 8 and 12 weeks). During the stipulated time period of experiment, none of the subject animals died or showed alterations in body weight, behaviour or general health. Further, the radiograph also showed intactness of the metallic implant (Fig. 1). Slight post-surgical inflammation was observed, which may be attributed to the body response towards surgery made for implantation. Metallic strips of all three SMAs, i.e. TNF1, TNF2 and TNF3, were found biocompatible, and no pronounced body reaction or effect on blood parameters and vital organs has been observed (Table 1). These SMAs contain alloying elements like iron, which might contribute towards higher concentrations of haemoglobin or can affect other blood parameters. No difference in haemoglobin concentration was observed between control and



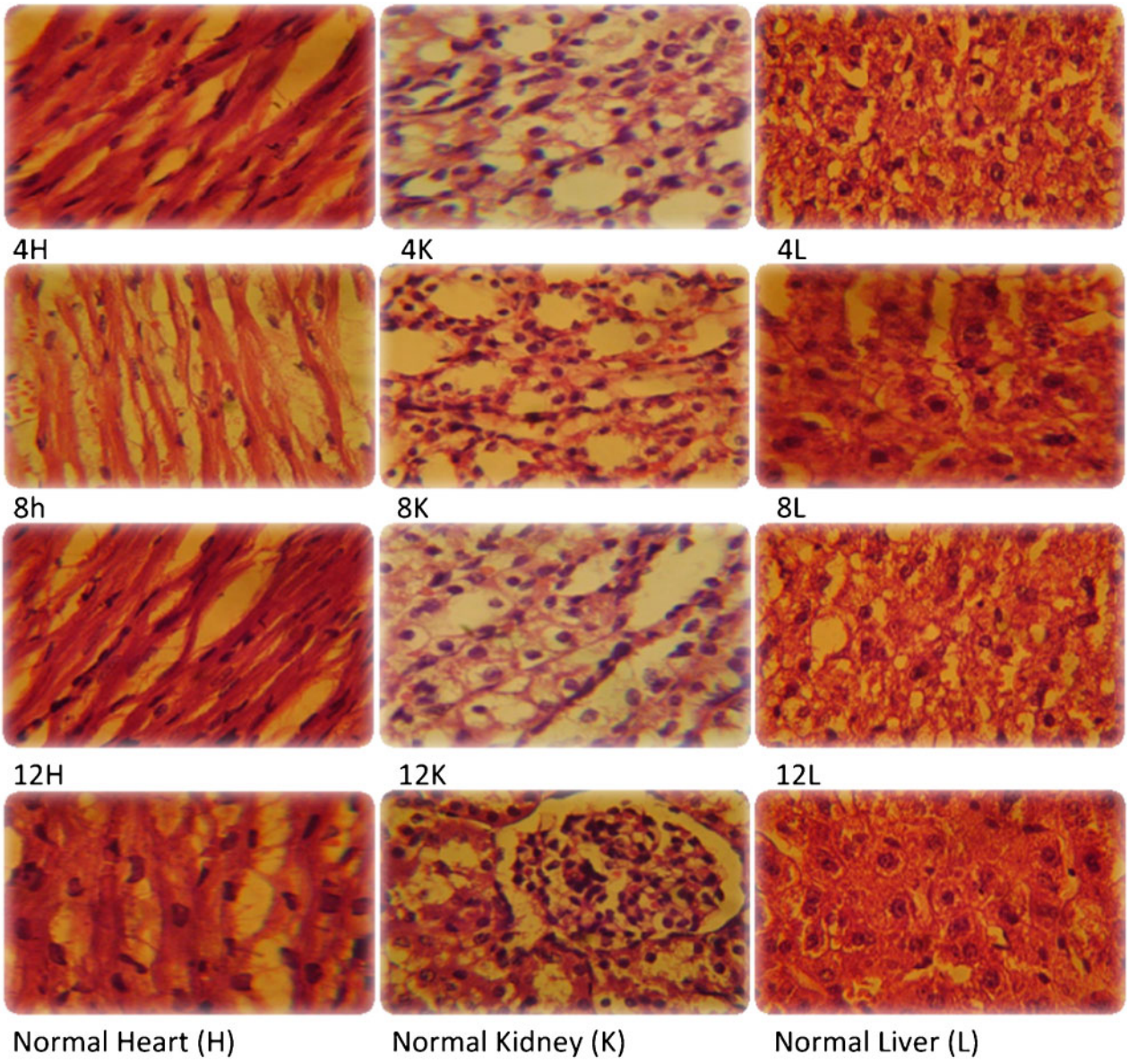
3 *In vivo* tissue response to alloy designated as TNF2 in rabbits of three groups showing vital organ histopathology [liver (L), kidney (K) and heart (H) at fourth, eighth and twelfth weeks post-implantation of TNF2 alloy]

treated animals, depicting that these implants have no systemic effects on blood parameters of subject animals (Table 1). Statistical analysis indicated that blood parameters of treated and control rabbits were not significantly different (Table 1). Systemic effect is a suitable method to evaluate alloys as biomaterials and may be cytotoxic at certain conditions.[12] It shows tissue response after the implantation of biomaterial.

The sectioning of vital organs (liver, kidney and heart) is to analyse if these heavy metal implants have any effect on normal working of these organs lying far away from site of implant or there is such clearance of toxic metabolites by liver and kidney. Furthermore, to elucidate cytotoxic effects and inflammatory response of body towards these metallic implants, tissues of vital organs such as liver, kidney and heart involved in metabolism and clearance of heavy metals from the body were histologically evaluated in treated animals and compared to control (Figs. 2–4). Thin tissue sections of liver, kidney and heart were stained with haematoxylin and eosin and visualised under a microscope. Tissue sections of the heart showed normal cardiac myocyte with elongated nucleus and minute presence of intercellular connective tissue, while tissue sections of kidney showed functional glomerulus having standard bowman's space. Renal tubules were healthy, and no proteinaceous exudates were found in lumen of tubules. However, in renal tissue section, no evidence of interstitial connective tissue was observed. Liver tissue sections showed eosinophilic stained cytoplasm having normal hepatocyte cord pattern, though a minute vacuolar degeneration was observed in all hepatic tissue including control group rabbit, representing it as a persistent lesion that shows non-significant change in relation to implanted alloy.

Conclusions

In the present study, the host response to Ti–Ni–Fe SMAs was within the acceptable range of cytotoxicity, and no toxic, irritating and inflammatory reaction was observed in treated and control animals, whereas histological study of vital organs like liver, heart and kidney tissues revealed no lesions like necrosis, granulomas or signs of dystrophic soft tissue calcification in animals implanted with Ti–Ni–Fe SMAs, depicting them to be safe and biocompatible to living system.



4 *In vivo* tissue response to alloy designated as TNF3 in rabbits of three groups showing vital organs histopathology [liver (L), kidney (K) and heart (H) at fourth, eighth and twelfth weeks post-implantation of TNF3 alloy]

Blood examination of treated and control animals revealed no specific reaction of these implants to biological system. All Ti–Ni–Fe SMAs show similar cytotoxicity and biocompatibility and may be designed as potential biomaterials for applications interfacing with biological system.

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