Arch Orthop Trauma Surg (1998) 118 : 126–130

ORIGINAL ARTICLE

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Changes of biochemical markers during fracture healing

Received: 15 February 1998

Abstract The aim of this study was to evaluate the changes of biochemical markers during fracture healing in patients with osteoporosis. The study included 26 patients; 9 underwent hip hemiarthroplasty (mean age ± SD: 71.0 ± 10.2 years, group EN) for femoral neck fractures, 7 underwent osteosynthesis (75.3 ± 8.2 years, group OS) for trochanteric fractures, and 10 subjects had spinal compression fractures (68.2 ± 12.0 years, group CO). No operative procedures were performed in group CO. Urinary pyridinoline (Pyr), deoxypyridinoline (Dpyr) by high performance liquid chromatography (HPLC), Crosslaps by both enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (CTx-ELISA and CTx-RIA) and serum *N*-terminal mid-fragment osteocalcin ($OC_{N\text{-}Mid}$) by ELISA were analyzed at the time of admission and at weeks 1, 2, 4, 8 and 24 after operation or, in the case of group CO, after admission. As a whole, bone resorption markers started to increase from week 1, with various peak values between weeks 4 and 8 depending upon the particular marker, but returned to the initial vales at week 24. $OC_{N\text{-}Mid}$ started to increase from week 8 and remained at elevated levels at week 24. In groups EN and OS, bone resorption markers changed in the same manner as they did as a whole group. $OC_{N\text{-}Mid}$ did not change in group EN, although it increased significantly from week 8 in group OS. No biochemical markers changed significantly in group CO. In conclusion, bone resorption was accelerated at an early stage due to acute osteonecrosis or bed rest, followed by bone formation due to callus or mechanical stress later on. As far as bone resorption markers are concerned, 24 weeks are enough to eliminate the effect of fracture.

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Introduction

The process of fracture healing can be divided into three distinct phases – inflammation, regeneration and remodelling. The changes of bone resorption and formation in this process are expected to be more dynamic than those changes which occur in the remodelling cycle alone because of aging.

Experimental animal models were developed for analysing the sequences of gene or protein expressions involved in the process of fracture healing [6, 11, 13]. Also serum or urinary biochemical markers were employed for evaluating the sequential changes of bone resorption and formation separately after tibial and radius fractures and after total hip or knee arthroplasty [7–9]. Since type I collagen constitutes about 90% of the organic matrix of bone and type III collagen is abundant in callus tissues, de novo synthetic products of type I and type III collagen in sera, as well as osteocalcin (which is the osteoblastic marker), have been measured in patients who sustained fractures or underwent arthroplastic operations. Urinary collagen crosslinks, pyridinoline (Pyr) and deoxypyridinoline (Dpyr), are currently available as useful bone resorption markers [15]. These two markers can distinguish osteoporotic patients with femoral neck fractures from those without fractures [10]. Recently, the urinary C-terminal telopeptide of type I collagen (CrossLaps, CTx) measured by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (the degradation product of type I collagen) and the *N*-terminal mid-fragment osteocalcin ($OC_{N\text{-}Mid}$) in serum, measured by ELISA, were developed [3, 4, 18].

In the present study, urinary Pyr, Dpyr, CTx-ELISA and -RIA, as bone resorption markers, and serum $OC_{N\text{-}Mid}$, as the sole bone formation marker, were measured sequentially in patients who sustained single spinal compression fractures or underwent operations for hip fractures. Conventionally, sera or urine are obtained immediately after or more than 6 months after the occurrence of hip fractures in order to eliminate the effect of the fracture on the biochemical parameters. Unfortunately, it is diffi-

cult to conclude that such a period – more than 6 months after a fracture – allows us to eliminate the effect of fractures on the biochemical parameters data. The aim of this study, therefore, is to evaluate the changes of bone resorption and formation markers separately during fracture healing and to determine at what point these fractures do affect biochemical markers in patients who sustained them.

Patients and methods

This study included 26 patients admitted to the Department of Orthopaedic Surgery at Iwata Municipal Hospital, Iwata, Shizuoka, Japan, for either femoral neck fractures or single vertebral compression fracture due to osteoporosis. Of these, 9 underwent cemented hip hemiarthroplasty [8 women and 1 man, aged 71.0 ± 10.2 (mean ± SD) years] for femoral neck fractures (group EN), and 7 underwent osteosynthesis with intramedullary flexible nails (Ender nail) or Gamma AP locking nail (Howmedica) (5 women and 2 men, aged 75.3 ± 8.2 years) for trochanteric fractures (group OS). The other 10 subjects were patients with single spinal compression fractures (5 women and 5 men, aged 68.2 ± 12.0 years) (group CO). No operative procedures were performed on the last group, and none of them showed neurological deficits. For 6 patients with hemiarthroplasty, Modular VS22 stem (3 M) with a standard acrylic polymethylmethacrylate (PMMA) bone cement was inserted, and an integral bipolar cup (3 M) replaced the femoral head during the operation. For the other patients with hemiarthroplasty, an HA/TCP coated stem (Zimmer) was inserted without bone cement, and a multipolar bipolar cup (Zimmer) replaced the femoral head. Patients with hip fractures who underwent cemented hemiarthroplasty and all patients with trochanteric fractures were allowed to try walking with or without a crutch 1 week after their operation. Patients who underwent hemiarthroplasty without bone cement were allowed to try walking only with support (partial weight-bearing) 6 weeks after their operation. Patients who suffered from spinal compression fractures were allowed to walk 1–2 weeks after admission depending upon their symptoms. One patient in the non-cemented hemiarthroplasty group had received 5 mg of prednisolone daily before admission because of myasthesia gravis. The other patients received no medicine that could have affected their calcium and mineral metabolism. In the operation group, all patients received daily doses of 2.0 g cefazolin sodium intravenously for 7–10 days, followed by daily doses of 1.5 g cefaclor orally for 3–4 weeks postoperatively.

All patients were admitted to hospital within 24 h after injury. Blood and urine samples were obtained at the time of admission. Thereafter, samples were collected 1, 2, 4, 8 and 24 weeks after the operation for hip fractures (or in the case of the CO group, 1, 2, 4, 8 and 24 weeks after admission). Samples were stored at –30 °C until analysis.

Urinary Pyr and Dpyr were measured by high performance liquid chromatography (HPLC) after acid hydrolysis using a Gilson ASPEC (automated sample preparation with extraction column) according to Pratt et al. [17]. This new method allowed us to measure urinary Pyr and Dpyr rapidly by eliminating the drying step which had been necessary when using the manual method. Furthermore, the data were more reliable since a synthetic pyridinoline derivative (acetyl Pyr) was used as the internal standard which did not interfere with the native Pyr or Dpyr peak on the HPLC chromatogram. In our protocol, the intra- and interassay coefficients of variation (CVs) were 6.4% and 5.9% for Pyr and 6.0% and 6.0% for Dpyr, respectively.

Urinary type I collagen degradation products (CTx-ELISA) were measured by the CrossLaps ELISA kit according to Bonde et al. [3]. The CTx-ELISA is based on an immobilized synthetic peptide with an amino acid sequence specific to part of the C-telopeptide of the CI chain of type I collagen (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg = CrossLaps peptide) The intra- and interassay CVs were less than 6% and 8%, respectively.

Urinary C-telopeptides of type I collagen (CTx-RIA) were also measured by a RIA based on a monoclonal antibody raised against an 8-amino acid sequence which was the same as the CrossLaps peptide according to Bonde et al. [4]. Within run and total CVs were 4.4% and 5.3%–6.2%, respectively, at concentrations of 1–7 mg/l. Values of urinary Pyr, Dpyr, CTx-ELISA and CTx-RIA were corrected for urinary creatinine concentration, which was measured on an autoanalyzer.

The serum concentration of osteocalcin ($OC_{N\text{-}Mid}$) was measured by a recently developed ELISA (two-site N-MID h Osteocalcin A/S, Denmark) [18]. The intra- and interassay CVs were less than 4.2% and 4.0%, respectively, and the detection limit was 2.0 ng/ml.

Statistical analysis

Two-way analysis of variance (ANOVA), followed by the Scheffe-F test for two related samples were used to evaluate differences. ANOVA followed by the Scheffe-F test was used for comparison between data in different groups. *P* values less than 0.05 were considered statistically significant. To eliminate the effect of the baseline values on subseeding longitudinal values, percent changes from the initial values were calculated at each time point.

Table 1 Anthropometric indices and initial values in each group (mean ± SE)

	Endo-prosthesis (EN)	Osteo-synthesis (OS)	Compression fracture (CO)
Number (M/F)	9 (1/8)	7 (2/5)	10 (5/5)
Age (years)	71.0 ± 3.4	75.3 ± 3.1	68.2 ± 3.8
Height (cm)	151.0 ± 2.0	154.4 ± 3.5	153.3 ± 5.6
Weight (kg)	49.6 ± 2.7	47.0 ± 5.0	52.7 ± 3.8
L2–4 bone mineral density (g/cm^2)	0.719 ± 0.062	0.787 ± 0.081	0.677 ± 0.034
Blood transfusion (ml)	333.3 ± 149.0	142.9 ± 71.0	0
Pyridinoline (nmol/mmol creatinine)	85.6 ± 33.5	72.5 ± 11.8	32.9 ± 6.2
Deoxypyridinoline (nmol/mmol creatinine)	17.3 ± 4.2	15.6 ± 2.7	7.3 ± 1.5
CrossLaps (CTx)-ELISA (μg/mmol creatinine)	373.9 ± 109.4	592.6 ± 136.7*	223.1 ± 42.4
CTx-RIA (μg/mmol creatinine)	501.6 ± 126.7	590.0 ± 124.9	283.5 ± 61.6
N-terminal mid-fragment osteocalcin ($OC_{N\text{-}Mid}$, (ng/ml)	17.0 ± 1.5	23.3 ± 7.2	13.7 ± 1.6

* $P < 0.05$ compared with compression fracture values

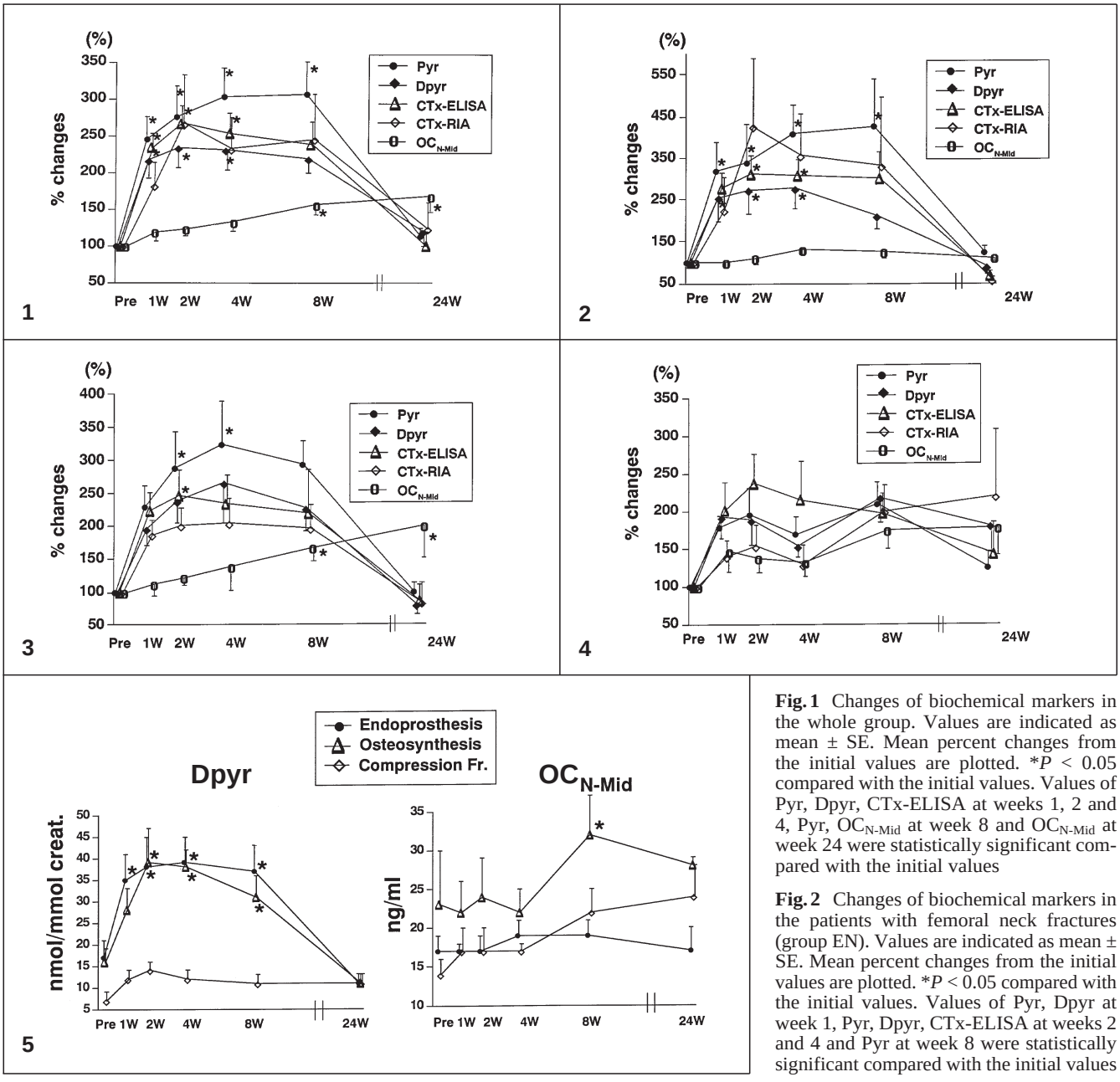

Fig. 1 Changes of biochemical markers in the whole group. Values are indicated as mean ± SE. Mean percent changes from the initial values are plotted. *$P < 0.05$ compared with the initial values. Values of Pyr, Dpyr, CTx-ELISA at weeks 1, 2 and 4, Pyr, $OC_{N\text{-}Mid}$ at week 8 and $OC_{N\text{-}Mid}$ at week 24 were statistically significant compared with the initial values

Fig. 2 Changes of biochemical markers in the patients with femoral neck fractures (group EN). Values are indicated as mean ± SE. Mean percent changes from the initial values are plotted. *$P < 0.05$ compared with the initial values. Values of Pyr, Dpyr at week 1, Pyr, Dpyr, CTx-ELISA at weeks 2 and 4 and Pyr at week 8 were statistically significant compared with the initial values

Results

The anthropometric data, volume of blood transfusion during the operation and the initial values of biochemical parameters in the three different groups are shown in Table 1. There were no significant differences in the anthropometric indices including age, height, weight and L2–4 bone mineral density (BMD) among the three groups. Although values of urinary Pyr, Dpyr, CTx-ELISA, CTx-RIA and serum $OC_{N\text{-}Mid}$ in groups EN and OS tended to be higher than those in group CO, no significant differences were observed except for urinary CTx-ELISA. Urinary CTx-ELISA in group OS was significantly higher than that in group CO.

Fig. 3 Changes of biochemical markers in the patients with trochanteric fractures (group OS). Values are indicated as mean ± SE. Mean percent changes from the initial values are plotted. *$P < 0.05$ compared with the initial values. Values of Pyr, CTx-ELISA at week 2, Pyr at week 4 and $OC_{N\text{-}Mid}$ at weeks 8 and 24 were statistically significant compared with the initial values

Fig. 4 Changes of biochemical markers in the patients with spinal compression fractures (group CO). Values are indicated as mean ± SE. Mean percent changes from the initial values are plotted

Fig. 5 Changes of urinary Dpyr (*left*) and serum $OC_{N\text{-}Mid}$ (*right*) in the three groups. Values are indicated as mean ± SE. *$P < 0.05$ compared with the values in group CO at the corresponding weeks (*left*). *$P < 0.05$ compared with the values in group EN at the corresponding weeks (*right*)

Percent changes of each biochemical parameter in the group as a whole are shown in Fig. 1. All four bone resorption markers started to increase 1 week after the operation or admission, with peak values at various times depending upon the particular bone resorption marker, and started to decrease from weeks 2 to 8. Urinary Pyr increased significantly at week 1 over initial values, reached its peak at week 8, then returned to the initial level at week 24. Urinary Dpyr and CTx-ELISA reached their peak values at week 2, and no significant differences were observed at week 8 compared their initial values. Serum $OC_{N\text{-}Mid}$ started to increase from week 8 and continued to increase during the observation period.

Percent changes from the initial values of biochemical parameters in each group are shown in Figs. 2–4. In group EN (Fig. 2), urinary Pyr and Dpyr increased significantly at week 1 compared with the pre-operative values. Urinary CTx-ELISA increased significantly at weeks 2 and 4 compared with pre-operative values. Urinary Pyr, Dpyr and CTx-ELISA returned to their initial values at week 24. Although serum $OC_{N\text{-}Mid}$ tended to increase, no significant differences were observed at any time postoperatively compared with the pre-operative values. In group OS (Fig. 3), urinary Pyr increased significantly at weeks 2 and 4 compared with the pre-operative values. Urinary CTx-ELISA increased significantly at week 2. Serum $OC_{N\text{-}Mid}$ tended to increase at week 1 and significantly increased at weeks 8 and 24 compared with the pre-operative values. In group CO (Fig. 4), no biochemical markers changed significantly, although serum $OC_{N\text{-}Mid}$ tended to increase after week 4.

To examine the specificity of each biochemical parameter for the fracture types, changes of urinary Dpyr and serum $OC_{N\text{-}Mid}$ in the three different groups are shown in Fig. 5. Levels of urinary Dpyr at weeks 2, 4 and 8 in groups EN and OS were significantly higher than those in the corresponding weeks in group CO (Fig. 5, left panel). Levels of serum $OC_{N\text{-}Mid}$ at week 8 in group OS were significantly higher than those at week 8 in group EN (Fig. 5, right panel).

Discussion

Longitudinal changes of biochemical markers after fractures or operations have been reported in several papers, mainly by Joerrings et al. [7–9]. They examined the changes of bone turnover by measuring type I or III collagen de novo or breakdown products in patients with tibial and radius fractures and in patients who had undergone knee or hip arthroplasty. Although our protocol was similar to theirs, more specific markers of bone resorption and formation were used separately to evaluate the changes of bone turnover after fractures in patients with osteoporosis.

In the group as a whole, bone resorption markers and the sole formation marker changed in different manners after fractures. All bone resorption markers except CTx-RIA increased dramatically by week 1 after the operation or admission. This increase of bone resorption markers reflected the acute osteonecrosis during the operation (group EN) or the physical unloading due to bed rest [12]. The time (at week 8) when Pyr reached its peak values was later than when Dpyr and CTx-ELISA did. Dpyr and CTx-ELISA changed downward at week 4 and did not differ significantly over initial values at week 8. Moreover, percent changes of peak values of Pyr were larger than those of Dpyr and CTx-ELISA. Pyr is a less specific marker of bone resorption than are Dpyr and CTx since it is abundant in almost all connective tissues. However, Dpyr is distributed mainly in bone and dentin, and 90% of the bone matrix consists of type I collagen [5]. The changes of urinary Pyr might reflect not only fracture repair, but also soft connective tissue repair, including that of muscle, hip joint capsule and subcutaneous tissue damage.

A two-site ELISA kit for measuring $OC_{N\text{-}Mid}$ recognizes both a large *N*-terminal mid-fragment and an intact molecule of osteocalcin. $OC_{N\text{-}Mid}$ had several advantages for clinical use over intact OC with regard to the variation of measurements (%CVs) and the stability for storage [19]. In this study, osteocalcin increased gradually from week 1 after the operation or admission and was significantly elevated at week 8. Usually, callus at the fracture site became visible on X-ray films 2 or 3 weeks after fracture. This slight increase of $OC_{N\text{-}Mid}$ at weeks 1 and 2 after fracture could reflect callus formation at the fracture site or skeletal unloading due to bed rest [16]. A significant increase of $OC_{N\text{-}Mid}$ at week 8 reflected the accelerated bone remodelling at the fracture site. It was unexpected that serum $OC_{N\text{-}Mid}$ reached its peak value at week 24. Serum levels of $OC_{N\text{-}Mid}$ measured immediately after femoral neck fractures (within 18 h) were significantly lower than those in age-matched controls [1], increasing by 44% after 4 months, as reported by Åkesson et al. [2]. This increase is significant compared with initial values 60 days after fracture, as reported by Obrant et al. [14]. Although it is unclear that the initial values of OC were lower than those in the age-matched controls, our results reflected theirs in a satisfactory manner. So far, no papers have reported longitudinal changes of OC after femoral neck fractures or vertebral fractures in patients with osteoporosis with a 24-week-follow-up. A significant increase of $OC_{N\text{-}Mid}$ found in this study at week 24 was interpreted as reflecting the later stage of osteoblasts in the differentiation or mechanical stress caused by walking or as though physical activity promotes osteoblast differentiation. At week 4, bone resorption markers changed downward, while the bone formation marker changed upward. These contradirectional changes of biochemical parameters indicate that the calcium balance of bone at the fracture site changed from negative to positive in the process of fracture healing.

In group EN, three (Pyr, Dpyr, CTx-ELISA) of four bone resorption markers increased significantly compared with the initial values, while the bone formation marker did not change throughout the study. In group OS, two (Pyr, CTx-ELISA) of four bone resorption markers and the bone formation marker increased significantly over the initial values. Operation by means of an endoprosthe-

sis required femoral neck osteotomy and multiple reaming in the femoral shaft, while operation by means of osteosynthesis (Ender nail and Gamma AP interlocking nail) was less invasive to the bone. Callus formation at the fracture site may be visible after osteosynthesis, while it is not expected after endoprosthesis. Therefore, bone resorption markers more markedly increased in group EN than group OS and the bone formation marker increased only in group OS.

In group CO, the trend was for $OC_{N\text{-}Mid}$ to increase at a later period during the study, although this increase did not reach significant values compared with initial values. Callus formation or the consolidation of the fractured vertebral body could be detected on X-ray films at the later stage. Further study will be needed to see if single spinal compression fractures do in fact affect the biochemical parameters.

CTx-RIA is the recently developed bone resorption marker which recognizes the same antigen as CTx-ELISA. This marker correlated significantly with CTx-ELISA and distinguished well the postmenopausal women from the premenopausal women [4]. In this study, CTx-RIA changed in a similar manner to other bone resorption markers. However, this change did not achieve significance, since the SE or SD of CTx-RIA was relatively larger than for other bone resorption markers.

In conclusion, bone resorption and bone formation markers reflected the bone remodelling process during fracture healing. They changed differently depending upon the fracture or operation types. As far as bone resorption markers are concerned, 24 weeks represent a sufficiently long period to eliminate the effect of fracture.

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