Effect of Long-Term Oral Warfarin (Marevan) on the Long Bone of Adult Male Albino Rat

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Abstract: Introduction: Warfarin is a widely worldwide used oral anticoagulant for various purposes either therapeutic or prophylactic which antagonize vitamin K and alter carboxylation of various bone proteins. Long term use of warfarin is an essential step in patients' treatment course ranging from months to years even for life in some congenital coagulation defects like protein c and s deficiencies**. Aim of the Work:** To study the histopathological changes that occur during long term administration of oral warfarin therapy on the long bone (femur) of adult male albino rat. **Materials and Methods:** Forty rats were studied. After two weeks of acclimatization, The animals were randomized into a control group and three treatment groups. The first group (A) was a control group. The second group (B) was treated with daily Oral warfarin (0.20 mg/kg). The third group (C) was treated with daily Oral warfarin (0.25 mg/kg). The fourth group (D) was treated with daily Oral warfarin (0.30 mg/kg). Three rats from each group sacrificed on days 1, 15 and four rats on day 30. Hematoxylin and eosin slides were obtained and examined for pathological changes under light microscope. Bonehisto-morphometry was performed on a region of 1 mm below the epiphyseal growth plate, which included the entire metaphysis, was subjected to light microscopy then using Image J software analysis computer program providing following six parameters; Thickness of both cortical and woven bone. Percent of total bone from total area. Thickness of osteoid surface. Osteoblast as well as osteoclast cell number per bone surface %. These parameters' results compared among the different experimental groups in relation to dose and duration of treatment then represented with stacked columns charts. **Results:** The results of the present work showed that long term use of warfarin specially with higher doses and longer durations causes pathological osteoporotic changes in femur bones which should raise concern regarding its long-term human use. **Conclusion:** From the present study it could be concluded that warfarin has considerable bony osteoporotic changes proportional to dose and duration of treatment so physicians should schedule a screening and prophylactic measures to prevent osteoporotic changes specially in old ages to lower the incidence of sudden pathological (osteoporotic) fractures.

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Key Words: Osteoporosis, Oral anticoagulant, Warfarin, long bones, proximal femur.

1. Introduction

Warfarin is a traditional oral anticoagulant used for stroke prevention in patients with nonvalvular atrial fibrillation (NVAF). It inhibits the activation of bone matrix proteins. Several studies have reported the possible link between warfarin use and an increased risk of osteoporotic fracture.**(Lau, et al.,2017)**. Although the most common side-effect associated with the use of warfarin is bleeding, other non-hemorrhagic side-effects has been reported. Warfarin use in pregnancy remains problematic. Deleterious effects on bone are also suggested by warfarin's ability to induce embryopathy (nasal hypoplasia, stippled epiphyses and distal extremity hypoplasia) if administered between six and 12 week gestation it causes fetal loss and stillbirth later in pregnancy. Warfarin crosses the placenta, placing the vitamin K-deficient fetus at risk of hemorrhage. In some patients, low doses may also result in sub-therapeutic anticoagulation. Heparin, an alternative to warfarin, does not cross the placenta1 but results in higher rates of thromboembolic complications. **(Kariv, et al.,2018)**. Warfarin is thought to interfere with clotting factors by inhibiting the C1 subunit of the vitamin K epoxide reductases enzyme complex, thereby reducing the regeneration of vitamin K1 epoxide. **(Kaminsky and Zhang,1997)**. The issue of whether long-term warfarin therapy results in decreased bone density is controversial, warfarin and other vitamin K antagonists have been the only class of oral anticoagulants available for atrial fibrillation to reduce the risk of stroke. However, their use has been limited by a narrow therapeutic range that necessitates frequent monitoring and dose adjustments resulting in substantial risk and inconvenience.**(Ruff, et al.,2014)**. There are three typical Vitamin K dependent proteins: osteocalcin (OC), matrix glutamic acid protein (MGP), and growth arrest specific protein 6 (Gas-6) which

play key functions in maintaining bone strength, vascular calcification inhibition, and cell growth regulation, respectively. On the other hand, warfarin prevents the activation of MGP and Gas-6; therefore, long-term use of warfarin is reported to be associated with osteoporotic fractures. **(Namba, et al.,2015)**. Osteoporosis is the most frequent metabolic bone disease, became a public health problem because of the increase of the average life span and increase of the disease incidence. Osteoporosis is characterized by low bone mass and architectural deterioration of the bone tissue together with the subsequent increase of the bone fragility and fracture predisposition. The decrease of the bone mass is the result of an imbalance between the processes that control the preservation of the skeletal mass. **(Marcu, et al.,2011)**

2. Material and Methods

Animals and drug Used:

Specific white albino male rats, weighing 155–189 g purchased from animal house of Faculty of pharmacy Mansoura university.

Warfarin (Marevan1mg tablets) was purchased from Tarshoby Pharmacy in Mansoura manufactured by Glaxo Smith Kline (GSK) Egypt with patch number A522900. All experiments werecarried out according to the guidelines of the Institutional Animal Ethics Committee.

Experimental Design:

Oral warfarin tablets grinded with distilled water to make a suspension of 1mg/25ml given orally on a constant daily time not related to meals by a Ryle after shaking well with doses according to Paget formula we multiply the dose of human by 0.018 to convert human dose to rat dose (mg/200gm weight) = 3 X 0.018 = 0.054 X 5 = 0.27 mg /Kg.**(Paget and Barnes,1964)**

After two weeks of acclimatization, the rats randomly divided into four groups (10 rats each):

- The first group (A) was a control group.
- The second group (B) was treated with daily Oral warfarin (0.20 mg/kg).
- The third group (C) was treated with daily Oral warfarin (0.25 mg/kg).
- The fourth group (D) was treated with daily Oral warfarin (0.30 mg/kg).

Three rats from each group sacrificed on days 1, 15 and four rats on day 30.

Specimens collection and processing:

The obtained femurs were subjected to fixation then washed and decalcified, dehydrated, paraffin coated then a4µm sections obtained for staining by H & E. Bone histo-morphometry was performed on a region of 1 mm below the epiphyseal growth plate, which included the entire metaphysis then using Image J software analysis computer program providing following six parameters:

- Thickness of both cortical and woven bone.
- Percent of total bone from total area.
- Thickness of osteoid surface.
- Osteoblast as well as osteoclast cell number per bone surface %.

3. Results and Discussion

Bone histomorphometry:

Statistically comparing different groups with same duration of treatment (24 Hrs.) but different warfarin doses showed that rats' sections treated with the highest dose have relative increase in the following parameters compared to other groups:

- Cortical bone thickness
- Osteoblast cell number/bone surface %
- Osteoclast cell number /bone surface %

Associated with relative decrease of the following parameters:

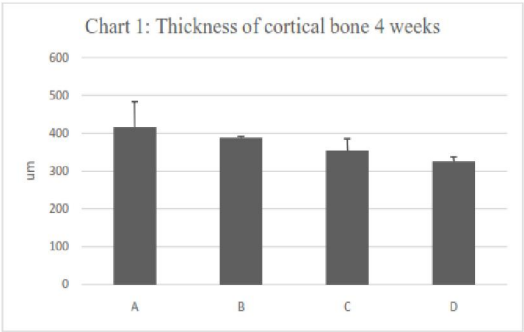
- Thickness of woven bone
- Percent of total bone to total area

With longer duration (2w and 4w) of treatment statistics comparing different groups with longer duration of treatment (2w and 4w) but different warfarin doses showed that rats' sections treated with the highest dose have relative increase in the following parameters compared to other groups:

- Osteoclast cell number /bone surface %

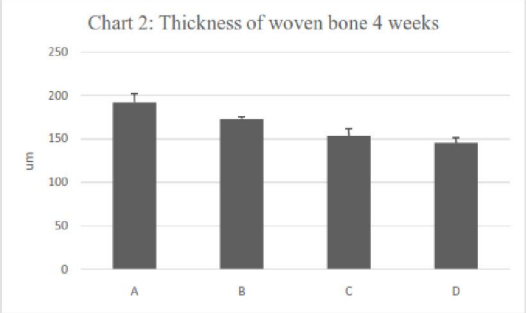
Associated with decrease of the following parameters:

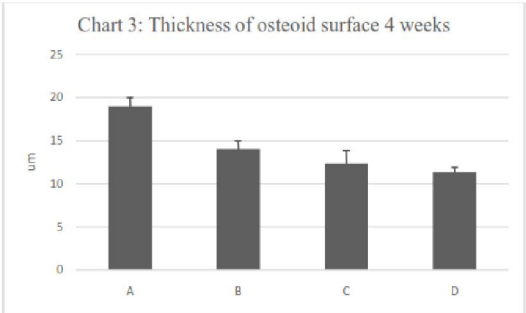
- Cortical bone thickness
- Thickness of woven bone
- Percent of total bone to total area
- Thickness of osteoid bone
- Osteoblast cell number /bone surface %

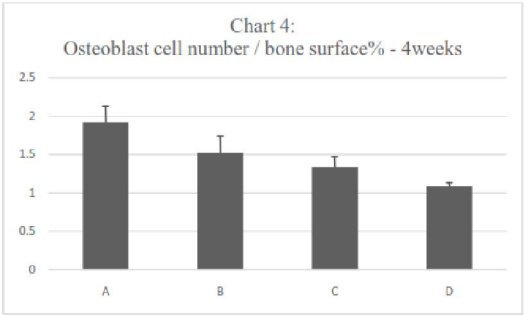


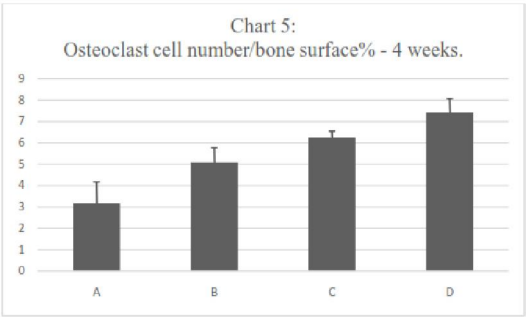
These results agree with (Simon, et al.,2002) who reported that IV sodium warfarin causes: (1) both a time and dose dependent decrease in femoral bone strength (2) a significant reduction in cancellous bone volume (3) a significant reduction in both

osteoblast number and activity and (4) a significant increase in osteoclast number.









Agree with (Caraballo, et al.,1999) informed that a significant increase in fracture incidence was reported in both the ribs and vertebrae of patients on long term warfarin therapy.

Disagree with (Woo, et al.,2008) reported in their cohort of community older men that no association was found between a single assessment of current warfarin use and bone mass (total hip or total spine), rates of hip bone loss, or risk of non-spine fractures.

Histopathological changes:

In group B

Rats treated with lowest warfarin dose for short periods of treatment (24hr) have minimal unremarkable bone changes.

However, rats subjected to longer duration of treatment (2w) shows thin cortical bone layer, mild osteopenia within the cortical layer, separate and short trabecular bones, increase fat tissue and few necrotic areas within the marrow.

Regards to longest duration of treatment in our study (4w) sections show marked changes in form of loss of osteoid tissue within the cortical and trabecular bone and marked depletion of haemopoietic tissue.

These results agree with (Lau, et al.,2017)reported that A total of 95 of 10279 patients on long term warfarin therapy developed osteoporotic fracture during follow-up.

These results also agree with (Gage, et al.,2006)who concluded that long-term use of warfarin was associated with osteoporotic fractures, at least in men with atrial fibrillation.

Disagree with (Misra, et al.,2014)reported in their large population-based cohort that they did not find any significant association between incident long-term warfarin use and risk of hip, spine, or wrist fractures, suggesting that warfarin use itself may not necessitate increased surveillance or prophylactic therapy for osteoporosis in elders on long-term therapy.

Disagree with (Woo, et al.,2008)reported in their cohort of community older men that no association was found between a single assessment of current warfarin use and bone mass (total hip or total spine), rates of hip bone loss, or risk of non-spine fractures. Including men who took osteoporosis medications at baseline or including excessive traumatic fractures did not alter these results, nor did the results differ according to level of dietary vitamin K intake.

In group C

Rats treated with warfarin dose of (0.25mg/kg) for short periods of treatment (24hr) have mild osteopenia with few fat cells.

However, rats subjected to longer duration of treatment (2w) shows separate tiny spicules of trabecular bones, marked loss of the compactness of the cortical layer, marked loss of the osteoid matrix within the cortical layer, absence of the trabeculae, necrobiotic changes within the bone marrow, microfracture features and decrease the thickness of the cortical layer.

Regards to longest duration of treatment in our study (4w) sections show marked changes in form of marked decrease of the osteoid matrix within the trabecular bone, marked osteopenia, very small-sized bone trabeculae within the bone marrow, thin cortical layer, decrease the trabecular bone areas, marked decrease of the osteoid matrix.

In group D

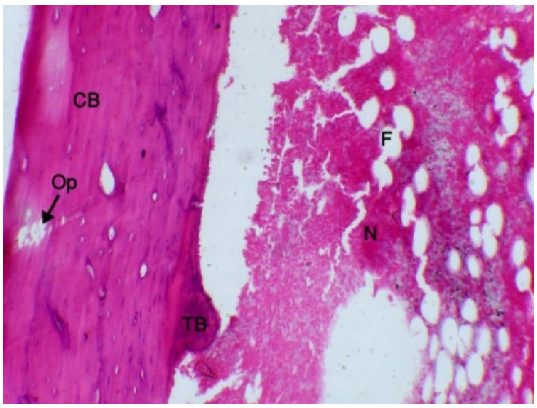


Fig. 1. Bone section of animal treated with oral Warfarin at dose of 0.20 mg/kg and sacrificed after 2 weeks post-treatment showing remarkable osteopenia (Op) within the cortical layer (CB), too short trabeculae (TB) and increase necrosis (N) and fat (F) within the marrow, (H & E, X200).

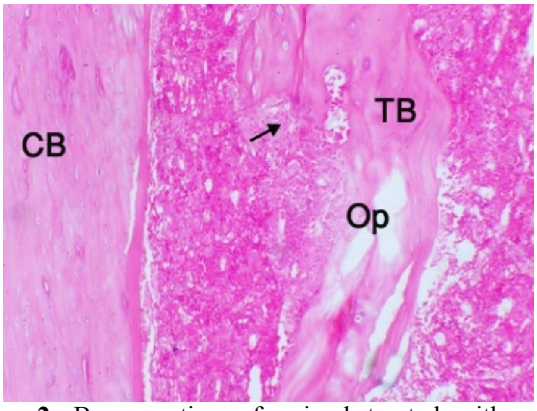


Fig. 2. Bone section of animal treated with oral Warfarin at dose of 0.30 mg/kg and sacrificed after 2 weeks post-treatment showing appearance of large resorption cavity (arrows) associated with marked osteopenia (OP) within bone trabeculae (TB) (CB indicates cortical bone), (H & E, X200).

Rats treated with highest warfarin dose (0.30mg/kg) for short periods of treatment (24hr) have moderate changes in form of thinning of cortical bone layer associated with osteopenia, osteonecrosis within the cortical layer.

However, rats subjected to longer duration of treatment (2w) shows multiple resorption areas with tunnel formation within bone trabeculae forming large resorption cavities in some sections.

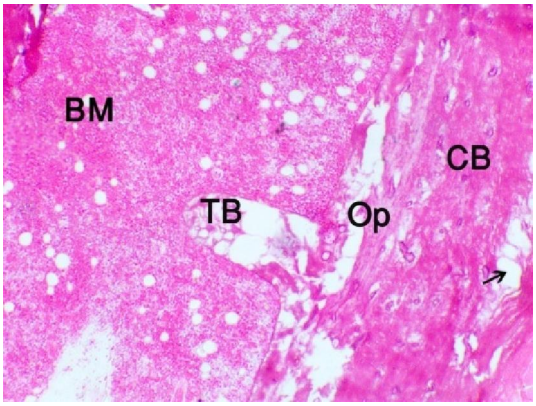


Fig. 3. Bone section of animal treated with oral Warfarin at dose of 0.30 mg/kg and sacrificed after 4 weeks post-treatment showing loss of the osteoid matrix (Op) of both trabecular and cortical bone (TB and CB respectively) associated with cavitation and tunneling of the cortical bone layer (arrow), (H & E, X200).

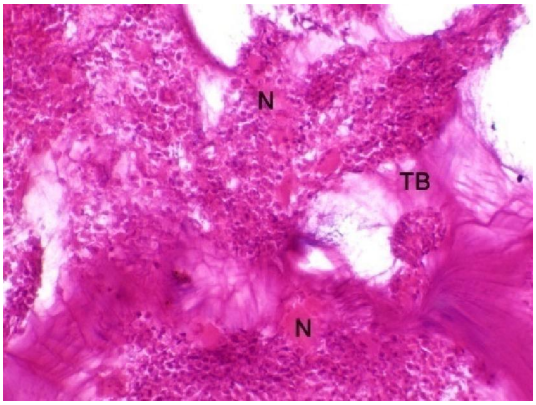


Fig. 4. Bone section of animal treated with oral Warfarin at dose of 0.30 mg/kg and sacrificed after 4 weeks post-treatment showing severe necrobiotic changes (N) within both trabecular bone (TB) and the bone marrow containing haemopiotic tissues, (H & E, X200).

Regards to longest duration of treatment in our study (4w) sections show loss of the osteoid matrix of both trabecular and cortical bone, cavitation and tunneling of the cortical bone, marked osteopenia, thin trabecular spicules associated with loss of the attachment with cortical layer, thin cortical layer, small tiny trabeculae within the bone marrow, marked loss of the osteoid matrix within the endostial surface of the cortical bone and severe necrobiotic changes.

These results agree with (Rezaieyazdi, et al., 2009) who found that treatment with warfarin

resulted in a significant decrease in lumbar spine bone mineral density.

Disagree with(Pilon, et al.,2004)reported that their study demonstrated a lack of significant association between oral anticoagulants and non-vertebral osteoporotic fracture despite of adequate sample size.

Disagrees withthe Jamal study(Jamal, et al.,1998)results showed that treatment with long-term warfarin have no effect on bone density or fracture incidence.

Conclusion

From the present study it could be concluded that warfarin has considerable bony osteoporotic changes proportional to dose and duration of treatment so physicians should schedule a screening and prophylactic measures to prevent osteoporotic changes specially in old ages to lower the incidence of sudden pathological (osteoporotic) fractures.

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