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Research Article

An investigation into the cellular-level adverse effects of tourniquet use on the infrapatellar fat pad in primary total knee arthroplasty: A prospective randomized study

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ABSTRACT

Objective: This study aimed to examine the cellular-level adverse effects of tourniquet use on the infrapatellar fat pad (IPFP) in patients undergoing primary total knee arthroplasty (TKA).

Methods: Infrapatellar fat pad samples were collected in a prospective, randomized design to compare 2 groups of primary TKA patients with a tourniquet (T) and without a tourniquet (NT). The study included 80 knees of 58 patients with a mean age of 65.91 ± 9.04 years. The authors collected 3 samples from the T group (after exposure to the fat pad "t1," just before deflating the tourniquet "t2," just before fascia closure "t3") and 2 samples from the NT group (t1 and t3) for each patient. BAX, Bcl-2, and HIF-1α staining showed the extent of cellular hypoxia and apoptosis in IPFP cells, whereas the oxidative stress index (OSI) was determined using a biochemical method. The Knee Injury and Osteoarthritis Outcome Score (KOOS), Knee Society Score (KSS), and Kujala score were used as clinical outcome measures.

Results: The mean HIF-1α, BAX/Bcl-2, and OSI scores across all time points were significantly higher in the T group than in the NT group ($p<0.001$) (d=1.16, 2.9, and 0.9, respectively). The mean BAX/Bcl-2 (P=.030) and HIF-1α (P < .001) scores significantly peaked at t2 in the T group (d=−1.2 and −3.9, respectively). The OSI had higher levels at t1 (P=.011) and t3 (P=.073) (d=0.2 and 0.1, respectively) than at t2 in the T group. The third-month postoperative follow-up revealed that the mean KOOS, KSS, and Kujala score improved significantly compared to the baseline preoperative values (P < .001); however, there was no difference between the T and NT groups regarding the maximum and total knee range of motion or clinical outcome scores.

Conclusion: Evidence from this study has shown that tourniquet use during primary TKA may be associated with significantly increased cellular hypoxia, oxidative stress, and apoptosis in the IPFP.

Level of Evidence: Level I, Therapeutic study.

Introduction

Tourniquets are widely used in total knee arthroplasty (TKA).[1,2] However, previous reports suggest that tourniquet application in TKA is associated with increased levels of oxidative stress and ischemia–reperfusion (IR) injury in the muscle tissue.[3,4] The literature suggests that a high concentration of free oxygen radicals produced by tourniquets in TKA is responsible for tissue damage.[3,5,6]

Cellular damage to the infrapatellar fat pad (IPFP) may be of clinical relevance because various studies have suggested that inflammation, necrosis, or subsequent fibrotic changes in the IPFP after trauma or surgery can lead to patellar tendon disorders, anterior knee pain, or various arthrofibrotic lesions.[7,8] These complications can lower patient satisfaction after TKA as a result of limited range of motion (ROM) and impaired quality of life.[9]

The amount of oxidative stress in various tissues can be qualitatively assessed using biochemical methods, and the immunohistochemical staining method can detect signs of IR injury and subsequent apoptosis at the cellular level, giving a snapshot of cells undergoing a dynamic process.[10-13] The biochemical method assesses the total oxidant status (TOS), total antioxidant status (TAS), and the ratio of TAS to TOS values, which is called the oxidative stress index (OSI). The OSI reflects the balance between oxidants and antioxidants.[10,11]

The primary aim of this study was to investigate the association between tourniquet application in primary TKA and the levels of oxidative stress (OSI), cellular hypoxia (HIF-1α), and subsequent apoptosis (BAX/Bcl-2) in IPFP cells using biochemical and immunohistological methods. We hypothesized that tourniquet use in primary TKA could induce hypoxia and associated apoptosis in the IPFP.

Materials and methods

Study design

A prospective randomized controlled study was conducted to evaluate IPFP samples from patients who were clinically diagnosed with advanced-stage

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osteoarthritis and Kellgren–Lawrence grade 4 radiological changes and underwent primary TKA with a tourniquet (T group) or without a tourniquet (NT group). These 2 groups were compared in terms of cellular hypoxia, oxidative stress, and subsequent apoptosis in the IPFP tissue. The study was conducted in the Orthopedics and Traumatology Department of Afyonkarahisar Health Sciences University after obtaining approval from the ethical committee of the same institution (dated May 3, 2019, numbered 2019/177). All the participants signed a detailed informed voluntary consent form.

Exclusion criteria for the study were intra-articular procedures that could affect the IPFP tissue, such as previous primary or revision knee arthroplasty and knee arthroscopy as well as conditions that could affect clinical outcomes after TKA, such as primary peripheral neurovascular disorders, ipsilateral musculoskeletal malignancies, sequelae due to trauma, and seronegative spondyloarthropathy. Patients meeting the eligibility criteria were randomized to the T and NT groups.

Randomization and blinding

Patients were randomized to the T and NT groups using a sequentially numbered, sealed, opaque envelope technique. The sequence was unknown to all participants. This technique involves using envelopes containing the assigned treatment (primary TKA with T or NT) for each participant. Clinicians were given these randomly generated treatment allocations within sealed opaque envelopes, and if the patient consented to participate in the study, the envelope was opened and the patient was then offered primary TKA with T or NT.

Surgical technique and tissue sampling

The same surgeon with over 20 years of arthroplasty experience operated on 40 knees in each group. All knees in the study received a posterior stabilized cemented (Destiknee Knee System®, Maxx Orthopedics, Inc., Norristown, Pa, USA) knee prosthesis. Tourniquet pressure was set at 150 mmHg above systolic blood pressure for standardization in the T group, and the operative and tourniquet duration times were recorded for each patient. All knees had varus deformity; therefore, a standard anterior midline approach with medial parapatellar arthrotomy was performed, along with "partial" Hoffa excision. The surgeon routinely performed patellar eversion in all cases for better visualization. Electrocautery was not used as a precaution in areas adjacent to the IPFP because it may affect tissue properties.

In this study, samples were collected from the IPFP, which is an adipose tissue that typically contains fewer fibroblasts and immune cells such as macrophages, mast cells, and lymphocytes.[14] Previous in vitro and animal studies have reported that adipocytes in fat tissue are highly susceptible to ischemia.[15,16] Therefore, we obtained samples from the IPFP to detect possible cellular hypoxia, oxidative stress, and apoptosis. Another important reason for this preference is that sampling from the IPFP rather than from the muscle tissue did not entail the risk of any potential morbidity to the patient and thus raised no ethical concerns.

HIGHLIGHTS

- Tourniquet application in total knee arthroplasty is associated with increased levels of oxidative stress and ischemia-reperfusion injury. This study aimed to investigate the association between tourniquet use and the levels of oxidative stress in primary total knee arthroplasty.
- The results showed that the mean oxidative stress index scores as well as hypoxia and apoptosis parameters were significantly higher in the tourniquet group. The results showed significantly increased irreversible cellular damage (apoptosis) in IPFP even with relatively short-term (57 minutes) tourniquet use. However, no significant difference was found in the third postoperative functional scores and total knee range of motion.
- The results indicate that tourniquet application during primary total knee arthroplasty is associated with significantly increased cellular hypoxia, oxidative stress, and apoptosis in the infrapatellar fat pad.

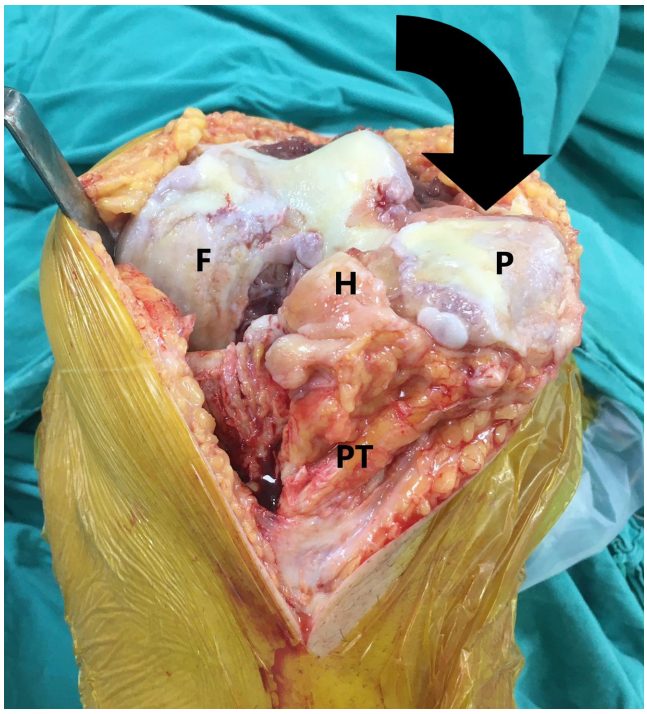

Figure 1. The figure shows infrapatellar fat pad after medial parapatellar approach and patellar eversion, before implantation. H, Hoffa'–s fat pad; F, femur condyle; P, patella; PT, patellar tendon.

In the T group, IPFP tissue samples were collected at 3 time points: after exposure of the fat pad (t1, approximately 5 minutes after tourniquet inflation), just before deflating the tourniquet (t2), and just before fascia closure (t3) (Figure 1). As there was no tourniquet in the NT group, samples were collected only at t1 and t3.

Immunohistochemical assessment

Immunohistochemical tests were conducted in the Histology Department of Afyonkarahisar Health Sciences University. After counting 500 cells for each sample, the histologist assigned 3 points to cells with the strongest staining intensity, 2 points for medium staining, 1 point for weak staining, and 0 for no staining. These values were then used to determine the *H*-score, which evaluates the total immunoreactivity of the tissue according to staining intensity using the following formula: (3 × number of strongly positive cells)+(2 × number of moderately positive cells)+(1 × number of weakly positive cells)+(0 × number of negative cells).[17]

BAX/Bcl-2 and HIF-1α secondary antibodies

BAX (Sc-526, Lot: G1414) and Bcl-2 (Sc-7382, Lot: H2813) secondary antibodies were used for immunohistochemical assessment of cell apoptosis (Figure 2). The extent of apoptosis was measured by the ratio of BAX to Bcl-2. In addition, HIF-1α (Neo Markers, Lot:1164P1205A) secondary antibodies were used as an indicator of cell hypoxia (Figure 2). Staining intensity for all markers was determined according to the *H*-score.

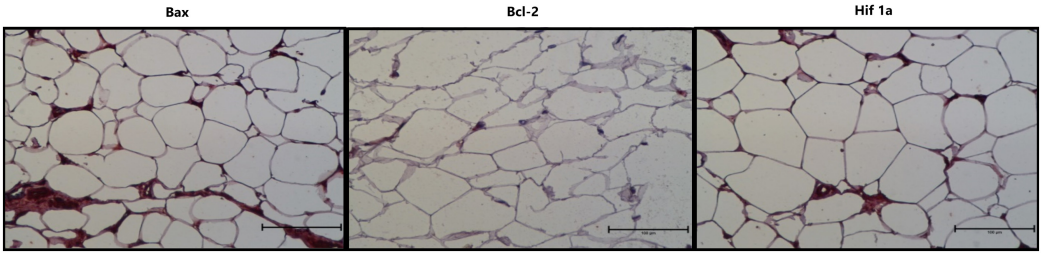

Figure 2. Immunohistochemical staining for BAX, Bcl-2, and HIF-1α antibodies in samples collected at t2 in the tourniquet group (×400 magnification, scale bar: 100 μm).

Biochemical assessment

Biochemical tests were conducted at the Biochemistry Laboratory of Afyonkarahisar Health Sciences University to determine levels of oxidative stress in the IPFP tissue. For each sample, TOS and TAS were determined and used to calculate OSI using the following formula: OSI=(TOS, μmol/L)/[TAS, (mmol TroloxEquiv/L] × 100.[11]

Clinical follow-up and outcome measures

The same follow-up regimen and rehabilitation protocol was used for all patients, with follow-up visits at 2 weeks, 1 month, and 3 months after surgery. Rehabilitation included mobilization on the second postoperative day and daily physiotherapy consisting of walking exercises, passive and active flexion/extension of the knee, strengthening of the lower limb muscles, and respiratory training. At the time of discharge from the hospital, the patients were instructed to perform a home exercise program.

Clinical outcome measures consisted of knee ROM, the Knee Injury and Osteoarthritis Outcome Score (KOOS), Knee Society Score (KSS), and Kujala score, all of which were assessed preoperatively and 3 months after primary TKA.

Statistical analysis

Categorical data were expressed as frequencies and percentages, and continuous variables were expressed as mean ± SD. The Mann–Whitney *U* test was used to compare the T and NT groups when parametric conditions were not met. The analysis of variance test was used to compare the means of 3 or more independent groups if parametric conditions were met. The independent-samples *t*-test and paired-samples *t*-test were used for parameters with a normal distribution to compare the preoperative and postoperative data of the T and NT groups. The Wilcoxon signed-rank and Friedman tests were used for parameters without a normal distribution. All data were entered and analyzed using Statistical Package for the Social Sciences Statistics for Windows version 22.0 (IBM SPSS Corp.; Armonk, NY, USA). The statistical significance level was set at $P < .05$. Relationships between numerical variables were evaluated using Pearson or Spearman correlation analysis. Correlation coefficients (*r*) were interpreted as follows: 0.00, no relationship; 0.01-0.29, weak relationship; 0.30-0.70, moderate relationship; 0.71-0.99, and strong relationship, 1.00, perfect relationship. Standardized effect sizes (Cohen's *d*) with 95% CIs were calculated as the difference between mean scores divided by the pooled SD.

Power analysis

Power analysis using the G-power computer program indicated that a total sample of 80 knees would detect significant effects ($d=0.8$) with 94% power using the Mann–Whitney *U* test between means with an alpha of 0.05.

Results

This prospective randomized controlled study included 80 knees of 58 patients [44 women (75.9%) and 14 men (24.1%)]. The flowchart

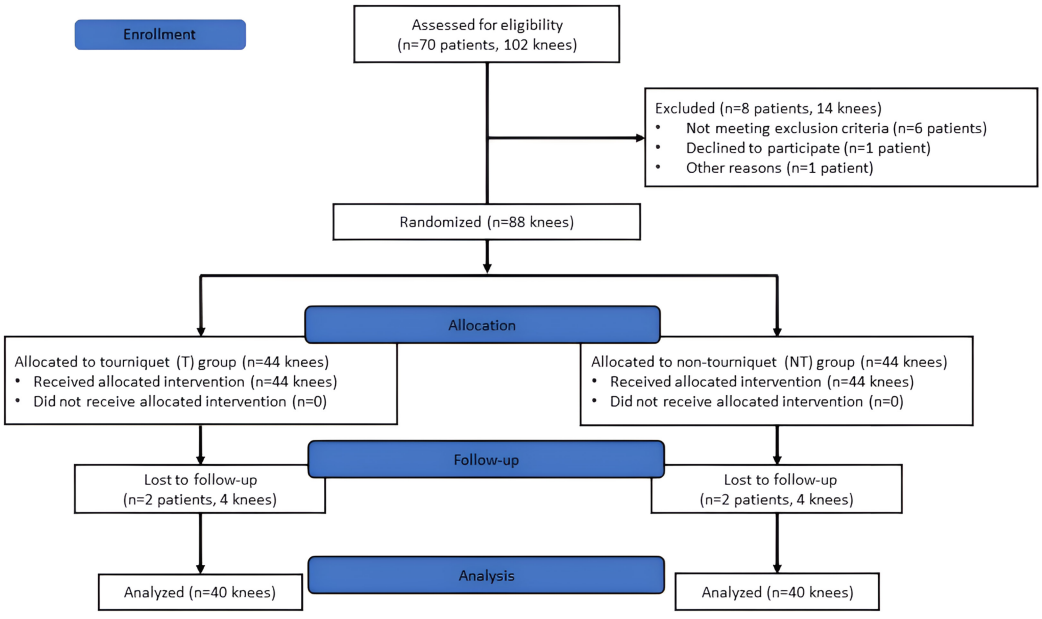

Figure 3. Study flowchart.

Table 1. Baseline demographic data

	T group	NT group	*P*
Age, years	64.92 ± 9.59	65.90 ± 7.89	.621
BMI, kg/cm²	33.16 ± 4.57	32.25 ± 4.69	.379
Obesity (BMI > 30 kg/cm²)	30	25	.233
Smoking, n	3	3	1
Diabetes mellitus, n	8	10	.578
Hypertension, n	27	25	.644
Coronary artery disease, n	2	3	.649

BMI, body mass index; NT, total knee arthroplasty without tourniquet; T, total knee arthroplasty with tourniquet.

of the study is presented in Figure 3. There was no significant difference between the groups in baseline demographic characteristics (Table 1) ($P > .05$).

Total knee arthroplasty with tourniquet group

The mean BAX/Bcl-2 scores in the T group fluctuated during surgery ($P < .001$) (Figure 4). The mean score for t2 samples was significantly higher than those for t1 and t3 samples (d=−1.2 and 2.5, respectively) (Table 2). The mean BAX/Bcl-2 score of samples collected at t3 (after tourniquet deflation) was significantly lower than that of samples collected at t1 (5 minutes after inflating the tourniquet) (d=−4.4).

The mean HIF-1α scores also fluctuated significantly during surgery in the T group (Figure 4). The mean HIF-1α score was significantly higher at t2 than at t1 (d=−3.9) and t3 (d=2.8) (Figure 4). Additionally, the mean HIF-1α score was significantly higher at t3 than at t1 (Table 2).

One might expect a lower HIF-1α score at t3 than t1. We attributed the discrepancy between t1 and t3 scores to the relatively longer half-life of HIF-1α and its subsequent latent reaction to hypoxia. We think that HIF-1α levels also continued to decline after t3 and fell below t1 levels, but we were not able to detect this within the scope of our study.

The mean OSI was significantly lower at t2 compared to t1 (d=0.2, P=.011). Although not significant, the mean OSI value increased between t2 and t3 (d=0.1, P=.073). The mean OSI of t3 samples was lower than that of t1 samples, but the difference was not statistically significant. (Figure 5).

Total knee arthroplasty without tourniquet group

There were no significant differences in the mean HIF-1α, BAX/Bcl-2, TOS, TAS, or OSI values of samples collected at t1 and t3 in the NT group ($P > .05$) (Table 2) (Figures 5 and 6).

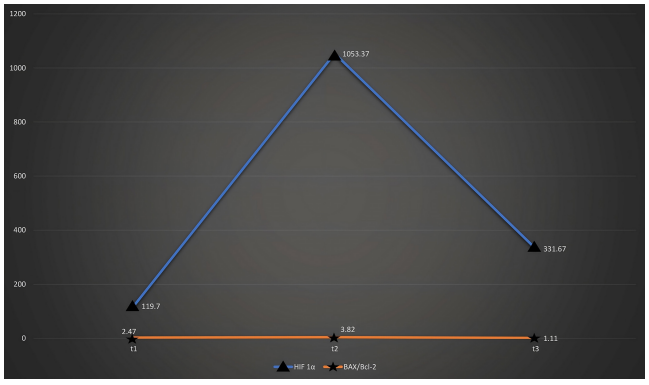

Figure 4. *H*-scores of HIF-1α and BAX/Bcl2 during surgery at t1, t2, and t3 in the total knee arthroplasty with tourniquet group. Note the significant fluctuation in HIF-1α during surgery and the peak at t2.

Table 2. Mean *H*-scores and OSI in the T and NT groups

	T group (n = 40)	NT group (n = 40)
HIF-1α		
t1	119.75 ± 82.17	335.30 ± 174.81
t2	1053.37 ± 327.27[a]	–
Mean differences	−933.6	–
95% CI	−038.3 to −829	–
t3	331.67 ± 149.85[b]	349.20 ± 165.12
Mean differences	−211.9	−13.9
95% CI	−263.5 to −160.4	−77.9 to 50.09
BAX/Bcl-2		
t1	2.47 ± 0.42	1.31 ± 0.36
t2	3.82 ± 1.53[a]	–
Mean differences	−1.35	–
95% CI	−1.88 to −0.81	–
t3	1.11 ± 0.13[b]	1.38 ± 0.33
Mean differences	1.35	−0.07
95% CI	1.22 to 1.49	−0.23 to 0.09
OSI		
t1	5.90 ± 4.15	2.87 ± 2.1
t2	3.71 ± 12.59[a]	–
Mean differences	2.19	–
95% CI	0.5 to 3.9	–
t3	4.85 ± 3.72	3.15 ± 3.19
Mean differences	1.05	−0.28
95% CI	−0.79 to 2.9	−1.5 to 0.97

BAX, BAX (Sc-526, Lot: G1414) secondary antibodies; Bcl-2, Bcl-2 (Sc-7382, Lot: H2813) secondary antibodies; HIF-1α, HIF-1α (Neo Markers, Lot:1164P1205A) secondary antibodies; NT, total knee arthroplasty without tourniquet; OSI, oxidative stress index; T, total knee arthroplasty with tourniquet; t1, ¯5 minutes after tourniquet inflation; t2, < 1 minute before tourniquet deflation; t3, 1 minute before fascia closure. [a]Statistically significant difference ($P < .05$) in t1 vs. t2 comparison within the group.[b]Statistically significant difference ($P < .05$) in t1 vs. t3 comparison within the group.

Total knee arthroplasty with tourniquet vs. without tourniquet groups

When averaged across all time points, the mean HIF-1α, BAX/Bcl-2, and OSI scores were significantly higher in the T group than the NT group (d=1.16, 2.9, and 0.9, respectively) (Table 3).

Tourniquet time

The mean operative time was 94.00 ± 20.97 minutes in the T group and 93.77 ± 14.65 minutes in the NT group (P = .956). The mean tourniquet time in the Tgroup was 57 minutes. Correlation analysis of tourniquet time and HIF-1α, BAX/Bcl-2, and OSI scores of t2 samples revealed no significant correlation between tourniquet time and HIF-1α (r=0.122, P=.452), BAX/Bcl-2 (r=0.216, P=.181), or OSI (r=0.171, P=.290).

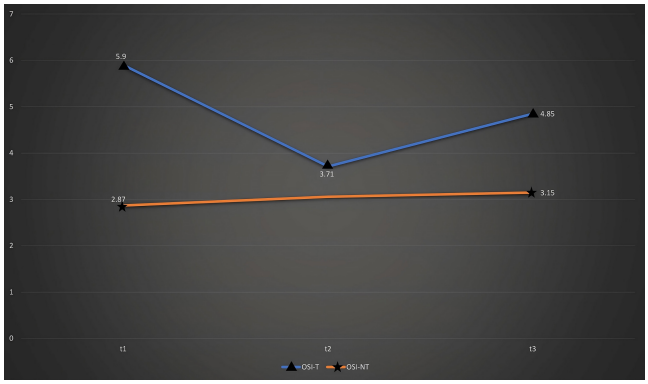

Figure 5. Change in OSI values in the T (t1, t2, and t3) and NT (t1, t2) groups during surgery. Note that inflating and deflating the tourniquet elicited acute and dramatic oxidative stress responses in the IPFP cells in the T group. IPFP, infrapatellar fat pad; OSI, oxidative stress index; NT, total knee arthroplasty without a tourniquet; T, total knee arthroplasty with tourniquet.

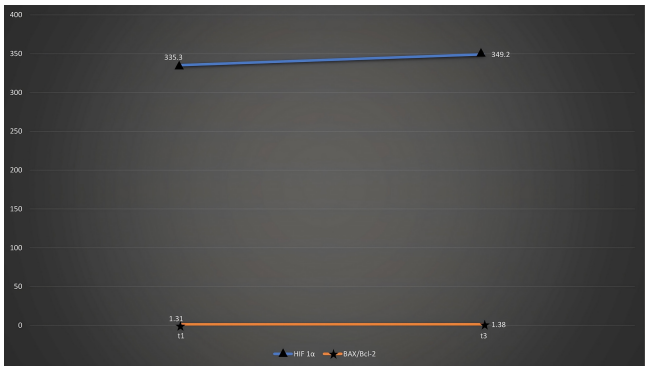

Figure 6. Changes in HIF-1α and BAX/bcl2 scores during surgery in the NT group. Note the insignificant difference in scores ($P > .05$). NT, total knee arthroplasty without a tourniquet.

Clinical outcome measures and physical examination findings

There was no significant difference in the mean maximum knee flexion between the preoperative and postoperative 3-month follow-up visits ($P > .05$). In contrast, the mean maximum knee extension and total ROM increased significantly between the preoperative and postoperative 3-month follow-up visits. Mean KOOS, KSS, and Kujala scores were significantly improved at postoperative 3 months compared to preoperative values. There were no significant differences between the T and NT groups in knee ROM or clinical outcome scores after primary TKA ($P > .05$).

Discussion

The results of this study revealed that the mean cellular hypoxia (HIF-1α), apoptosis (BAX/Bcl-2), and oxidative stress (OSI) levels were significantly higher in the IPFP of patients of the T group when compared with patients of the NT group. The mean cellular hypoxia score reached its maximum value before tourniquet release and cellular hypoxia persisted after tourniquet deflation. In addition, apoptosis was strongly induced by inflation of the tourniquet, reaching the highest level just before deflation, and decreased again after the release of the tourniquet. Thus, this study demonstrated irreversible cellular damage in the IPFP, even with relatively short-term (57 minutes) tourniquet use during primary TKA.

Several authors have suggested different methods or modalities, such as biopsy, magnetic resonance imaging (MRI), biochemical tests, or electromyography, to associate tourniquet use in surgery and cellular injury in muscle tissue.[4,18,19] To the best of our knowledge, this study is the first to investigate the adverse effect of tourniquet use in the IPFP at the cellular level using a randomized controlled design in primary TKA patients. The IPFP is typically an adipose tissue with fewer fibroblasts and immune cells such as macrophages, mast cells, and lymphocytes.[14] The rationale behind this preference is that previous in vitro and animal studies have reported that adipocytes in fat tissues are very susceptible to ischemia.[15,16] For instance, Eto et al[15] investigated a non-vascularized fat graft model and found that most adipocytes progressed toward cell death on the same day after exposure to ischemic conditions. Therefore, this study sought histological evidence of cellular hypoxia, oxidative stress, and apoptosis in IPFP tissues secondary to tourniquet use.

Previous studies have reported that IR injury typically occurs due to the oxygen-rich environment in the extremities resulting from a period of cellular hypoxia followed by tissue reperfusion after deflation of the tourniquet.[4,20,21] A systematic review analyzing 28 clinical studies suggested that tourniquets in TKA cause local IR injury in the skeletal muscle, increase protein destruction, and inhibit protein synthesis.[3] Likewise, the results of this study indicated significantly increased mean cellular hypoxia in the IPFP tissues of the T group. We think it is important to demonstrate cellular hypoxia in the IPFP because hypoxia, oxidative stress, and apoptosis were reported to be closely related in previous studies.[22,23]

This study also demonstrated significantly increased mean oxidative stress in the T group, indicating that tourniquet use increased oxidative stress in the IPFP related to inflation (ischemia) and deflation (reperfusion) of the tourniquet. Westman et al[24] reported that tourniquet use in TKA increased oxidative stress dramatically in the ischemic phase and late reperfusion phase at postoperative 24 hours.[3] Muyskens et al[4] collected quadriceps muscle samples from 13 patients who underwent tourniquet-applied TKA and similarly reported increased cellular stress induced by the tourniquet, particularly in the elderly.[4]

The results of this study demonstrated a significant increase in apoptosis in IPFP tissues associated with tourniquet use in primary TKA. Similarly, another study by Dreyer[25] reported an average muscle loss of 18% in older adults using MRI 6 weeks after tourniquet-applied TKA. These results suggest that using a tourniquet during TKA may cause increased permanent cell loss in adipose IPFP tissue owing to induced cellular apoptosis mechanisms, which may be of greater importance in the elderly population with reduced cellular regeneration capacity.

There are no strict guidelines on safe tourniquet duration. However, the consensus is that less than 2 hours of tourniquet use on the extremity is not associated with permanent injury.[25] This study had a mean tourniquet time of 57 minutes. The results of our correlation analysis suggested tourniquet duration may be positively associated with cellular hypoxia, apoptosis, and oxidative stress, although the relationships were not statistically significant. Different populations with longer mean tourniquet times may yield more meaningful results in the correlation analysis between tourniquet duration and adverse effects.

The postoperative KSS, KOOS, and Kujala scores were significantly higher in both of our study groups compared to baseline. However, functional and clinical outcomes and postoperative complications did not differ between patients who underwent TKA with and without a tourniquet. We attribute this insignificance to the short-term follow-up of 3 months in this study. It is known that irreversible cellular apoptosis induces fibrosis.[26] However, fibrosis and subsequent morphological changes take longer to occur. For instance, Weale et al[27] reported that significant changes in the patellar tendon could be observed 8 months after TKA and tended to persist for up to 5 years after the procedure.[28] Therefore, the authors think that the clinical implications of significantly increased cellular hypoxia, oxidative

Table 3. Comparison of mean *H*-scores and OSI between the tourniquet and non-tourniquet groups

	T group (n = 40)	NT group (n = 40)	*P*
Mean HIF-1α score	501.60 ± 137.33	342.36 ± 138.45	< .001
Mean BAX/Bcl-2 score	2.47 ± 0.49	1.34 ± 0.23	< .001
Mean OSI	4.82 ± 1.97	3.01 ± 1.88	< .001

BAX, BAX (Sc-526, Lot: G1414) secondary antibodies; Bcl-2, Bcl-2 (Sc-7382, Lot: H2813) secondary antibodies; HIF-1α, HIF-1α (Neo Markers, Lot:1164P1205A) secondary antibodies; NT, total knee arthroplasty without tourniquet; OSI, oxidative stress index; T, total knee arthroplasty with tourniquet.

stress, and apoptosis in the IPFP is relevant in the longer term. Additionally, injury and subsequent fibrosis in the IPFP have been reported to be associated with conditions such as persistent anterior knee pain, arthrofibrosis, patellar tendon disorders, and infrapatellar contracture syndrome, all of which can severely alter patient satisfaction after primary TKA.[7,8]

To the best of our knowledge, this study is the first to investigate the adverse effects of tourniquet use in the IPFP at the cellular level using a randomized controlled design in primary TKA patients. This study also used a combination of BAX, Bcl-2, and HIF-1α staining in addition to the biochemical marker of OSI to demonstrate signs of cellular hypoxia, oxidative stress, and apoptosis. However, this study had limitations. The study only examined the acute effects of hypoxia and reperfusion. It was not possible to compare the immediate findings with late-period changes because a late biopsy in the postoperative period was not possible due to ethical issues. Another limitation was that the inclusion and exclusion criteria were very specific. Therefore, the results of this study cannot be generalized to the entire population. The short follow-up period in this study is also a limitation. Long-term follow-up could reveal more information about the clinical implications of IPFP damage after using a tourniquet in primary TKA.

This study suggests that tourniquet application during primary TKA is associated with significantly increased cellular hypoxia, oxidative stress, and apoptosis in the IPFP. The results showed significantly increased irreversible cellular damage (apoptosis) in the IPFP even with relatively short-term (57 minutes) tourniquet use. Apoptosis is known to lead to fibrosis and subsequent structural changes in the tissues. Based on these findings, orthopedic surgeons may consider performing primary TKA without a tourniquet. Another option may be total excision of the IPFP rather than partial excision in order to avoid the fibrotic process and subsequent morphologic changes after increased cellular apoptosis, particularly in patients with relatively advanced age and prolonged tourniquet application.

Ethics Committee Approval: This study was approved by Ethics Committee of the Afyonkarahisar Health Sciences University (Protocol no: #2019/177).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – R.A., M.Y., Ö.Ö.; Design – R.A., M.Y., Ö.Ö.; Supervision – R.A., M.Y., Ö.Ö., F.F.; Materials – R.A., M.Y., Ö.Ö., Ç.K., S.Ş.; Data Collection and/or Processing – R.A., M.Y., Ç.K., S.Ş., F.F.; Analysis and/or Interpretation – R.A., M.Y., Ç.K., S.Ş., F.F.; Literature Review – M.Y., Ö.Ö.; Writing – M.Y.; Critical Review – R.A., Ö.Ö.

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