The Effect of Daily Fluoride Applications in Combination with Saliva Substitutes on Remineralization of Bovine Dentin and Enamel Subsurface Lesions.
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This Doctoral Thesis is based on the following original papers:


To my family
LIST OF CONTENTS

1. Introduction ............................................................................................................. 1
2. Objectives and Hypotheses .................................................................................... 3
3. Materials and Methods ............................................................................................ 4
   3.1. Specimens Preparation ...................................................................................... 4
   3.2. In vitro Storage and Treatment ........................................................................... 4
   3.3. Transversal Microradiography Analysis .............................................................. 4
   3.4. Statistical Analysis .............................................................................................. 5
4. Presentation of Original Manuscripts .................................................................... 6
5. Discussion ............................................................................................................... 7
6. Conclusions ........................................................................................................... 12
7. Abstract .................................................................................................................. 13
8. Zusammenfassung ................................................................................................ 14
9. References ............................................................................................................. 15
10. Acknowledgments ............................................................................................... 19
11. Curriculum Vitae ................................................................................................. 20
12. Eidesstattliche Erklärung ..................................................................................... 21
1. INTRODUCTION

The importance of saliva as a protective factor against tooth decay is well recognized (Dowd, 1999). Patients suffering from hyposalivation are considered a prominent risk group for tooth decay due to the rapid progression of dental caries in the absence of natural saliva and its remineralizing properties (Atkinson et al., 2005; Tschoppe et al., 2010). This relationship has been clearly demonstrated in patients irradiated for tumors in the head and neck areas. The quantitative and qualitative salivary changes predispose irradiated patients to a highly destructive form of dental caries which has a rapid onset and progression (frequently called "radiation caries") (Jensen et al., 2003; Vissink et al., 2003; Kielbassa et al., 2006a). Since the therapy of hyposalivation is generally restricted to palliative treatment, a rigorous supportive care is recommended to this patient group, including daily application of fluoride products in combination with meticulous oral hygiene and careful control of dietary intake (Vissink et al., 2004; Atkinson et al., 2005; Kielbassa et al., 2006a).

Moistening of the oral mucosa with saliva substitutes is a widely used alternative to relieve the symptoms of hyposalivation (Nieuw Amerongen and Veerman, 2003; Atkinson et al., 2005; Kielbassa et al., 2006a; Hahnel et al., 2009). A number of artificial salivas have been developed for palliative treatment of patients with salivary hypofunction. These products, based on hydrophilic polymers, act to replace or boost the salivary film (Urquhart and Fowler, 2006). In this regard, artificial saliva has proven to be at least partially effective in relieving the subjective symptoms of hyposalivation (Momm et al., 2005; Hahnel et al., 2009). From a dental point of view, it would seem reasonable to expect that saliva substitutes will not damage sound dental hard tissues. Moreover, since patients with extensive mouth dryness are particularly susceptible to dental caries, a remineralizing effect of the artificial saliva on initial lesions would be very desirable.

However, some saliva substitutes have shown demineralizing properties on dental hard tissues, and might be disadvantageous for dentate patients suffering from hyposalivation (Kielbassa et al., 2001; Smith et al., 2001; Meyer-Lueckel et al., 2002). Glandosane, a carboxymethylcellulose (CMC) based solution, is a commercially available product that revealed demineralizing effects on enamel as well as on dentin (Kielbassa et al., 2001; Smith et al., 2001; Meyer-Lueckel et al., 2002). In order to increase the inherent remineralizing capacities, inorganic substances such as fluorides, calcium, and phosphates have been added to saliva substitutes (Hahnel et al., 2009;
Tschoppe et al., 2009b). Saliva substitutes supersaturated with respect to octacalcium phosphate (OCP) should preferably be used, since calcium and phosphates may form complexes with the polymer ingredients of artificial salivas (Gelhard et al., 1983; Vissink et al., 1985). Recently, it has been shown that mucin and polysaccharide based saliva substitutes supersaturated with respect to OCP enabled remineralization of dentin and enamel (Meyer-Lückel and Kielbassa, 2006; Tschoppe et al., 2009b).

Although irradiated dentin was not more susceptible to caries than non-irradiated one (Kielbassa, 2000), dentin seems to be directly affected by radiotherapy (Kielbassa et al., 1997; Kielbassa et al., 2002). The effects of radiation on salivary glands and the radiation-induced damage at the dento-enamel junction can be considered the main causes of radiation caries in irradiated patients (Grötz et al., 1997; Kielbassa, 2000). Caries onset in these patients usually starts on the labial surface at the cervical areas of the teeth (Kielbassa, 2000; Kielbassa et al., 2006a). Therefore, efforts should be directed to the prevention of dentin lesion occurrence (Kielbassa et al., 2006a; Hahnel et al., 2009). However, salivary gland hypofunction may also be caused by other conditions such as organic diseases (e.g., diabetes) or autoimmune reactions (e.g., Sjögren’s syndrome), psychogenic factors, decreased mastication, and the use of medications with anticholinergic side effects (antihypertensives, antidepressants, analgesics, tranquilizers, diuretics and antihistamines). For this reason, attempts should also be directed to the prevention of enamel lesions.

The use of fluoride-containing products is considered an important requirement to caries prevention in patients with hyposalivation. Daily application of fluoride gel or mouthrinse in addition to the regular use of toothpaste has demonstrated a successful prevention of dental caries in irradiated patients (Meyerowitz et al., 1991; Spak et al., 1994; Papas et al., 2008). Notwithstanding this, only scanty information concerning the effects of saliva substitutes in combination with fluoride products on remineralization of initial lesions is available in the literature. Topical application of fluoride-containing products might be suitable to reduce demineralization of subsurface lesions in combination with a demineralizing saliva substitute, or to increase remineralization if used additionally with a remineralizing saliva substitute. Our intention with the in vitro studies presented in this thesis was to determine if daily fluoride applications (e.g., mouthwash and toothpaste) could be used in combination with saliva substitute as a promising approach for reducing or preventing dental hard tissue demineralization in patients with hyposalivation.
2. OBJECTIVES AND HYPOTHESES

Study 1 - The present study was undertaken to compare the effects of different experimental solutions (modified with respect to the degree of saturation of calcium phosphates, and with the main focus on precursors of remineralization: OCP) of a commercially available saliva substitute (Saliva natura; medac, Hamburg, Germany) in combination with highly concentrated fluoride toothpaste on the mineral loss and lesion depths of demineralized (subsurface lesions) bovine dentin in vitro. The null hypothesis claimed that slightly supersaturated solutions (with respect to OCP) in combination with highly concentrated fluoride toothpaste would not differ significantly in their remineralizing capacities.

Study 2 - The purpose of this study was to evaluate the effects of different saliva substitutes in combination with a mouthrinse and/or a high fluoride toothpaste on the mineralization of bovine dentin subsurface lesions. A commercially available and widely used saliva substitute (Glandosane; cell pharm, Hanover, Germany) was studied in comparison to an experimental and possibly remineralizing product (modified solution of Saliva natura, \( S_{OCP} = 2.0 \); medac, Hamburg, Germany). It was hypothesized (H0) that the additional use of fluoride products (mouthrinse or toothpaste) would not result in less pronounced demineralization of Glandosane, and that fluoride products would not result in enhanced remineralization of modified Saliva natura.

Study 3 - This in vitro study aimed to determine if daily applications of fluoride products (mouthrinse and/or high fluoride toothpaste) could influence the remineralization of subsurface bovine enamel lesions stored in two saliva substitutes. The null hypothesis tested was that daily applications of fluoride products in combination with 1) a known demineralizing saliva substitute (Glandosane; cell pharm, Hanover, Germany) would not result in significantly less pronounced demineralization; and 2) a possibly remineralizing artificial saliva (experimental Saliva natura, supersaturated with respect to OCP; medac, Hamburg, Germany) would not result in significantly enhanced remineralization.

These null hypotheses were tested against the alternative hypothesis of a difference.
3. MATERIALS AND METHODS

3.1. Specimens Preparation
Freshly extracted permanent bovine central incisors were used to prepare dentin and enamel specimens (6 × 4 × 4 mm³). From each crown three enamel slabs were prepared from the labial aspects, and four dentin slabs were obtained from the root cervical areas using a diamond-coated band saw under continuous water cooling. The specimens were embedded in epoxy resin with maximum caution to keep the natural surface free from resin. The surfaces were ground flat and hand-polished up to 4000 grit. One-quarter of each polished specimen surface was covered with an acid-resistant nail varnish to serve as control of sound tissue. Subsequently, specimens were immersed in a demineralizing solution to create subsurface lesions (pH 4.95 for enamel and 5.0 for dentin). The enamel specimens were demineralized in an incubator (37 °C) for 10 days, whereas the dentin specimens were demineralized for 5 days.

3.2. In vitro Storage and Treatment
Prior to the specimens’ treatment, the demineralized surface was partially covered with nail varnish to obtain a demineralization control within the same specimen. According to the study protocol, specimens were randomly allocated to different groups based on the storage solution. The degree of saturation (S) with respect to calcium-containing compounds (i.e., dicalcium phosphate dihydrate, DCPD; octacalcium phosphate, OCP; hydroxyapatite, HA; and calcium fluoride, CaF₂) was calculated for all aqueous solutions used, since the pH and the concentrations of certain ions were known (Shellis, 1988). Specimens were stored in the various solutions for 5 weeks (37 °C). During this period, specimens were submitted to different fluoride treatments. After each treatment, specimens were washed for 20 seconds with deionized water. The storing solutions were replenished every two days. After 2 weeks, half of the exposed surfaces (1.5 × 4 mm²) of each specimen were additionally varnished (effect after 2 weeks).

3.3. Transversal Microradiography Analysis
After in vitro storage and treatment, the investigator was blinded to prevent any bias. Thin sections (100 µm thick) were prepared from each specimen, and contact microradiographs (TMR) were obtained (Meyer-Lueckel et al., 2002; Tschoppe et al., 2009a). During radiography procedures the dentin specimens were treated with
ethylene glycol to avoid shrinkage (Ruben and Arends, 1993). A dedicated software was used to calculate the mineral loss ($\Delta Z$, vol% × µm) and lesion depth (LD, µm) of each area within the same specimen (sound, demineralized, effect after 2 and 5 weeks). Mineral losses and lesion depths of the demineralized ($\Delta Z_{\text{Demin}}$, $L D_{\text{Demin}}$) and effect areas ($\Delta Z_{\text{Effect} 2}$/LD$_{\text{Effect} 2}$; $\Delta Z_{\text{Effect} 5}$/LD$_{\text{Effect} 5}$) were corrected by subtraction of the respective sound control area values ($\Delta Z_{\text{Sound}}$, $L D_{\text{Sound}}$). Changes in mineral losses ($\Delta \Delta Z_{\text{Effect}} = \Delta Z_{\text{Demin}} - \Delta Z_{\text{Effect}}$) as well as changes in lesion depths ($\Delta LD_{\text{Effect}} = L D_{\text{Demin}} - L D_{\text{Effect}}$) were calculated for 2 and 5 weeks. Positive $\Delta \Delta Z$ or $\Delta LD$ values were considered as remineralization, and negative results were defined as demineralization.

3.4. Statistical Analysis

Statistical analyses were performed using a statistical software package (SPSS for Windows, version 11.5). Data were tested for normal distribution (Kolmogorov-Smirnov test). Analysis of variance (one-way ANOVA) followed by Tukeys’ post-hoc test was used to evaluate differences for $\Delta \Delta Z$ and $\Delta LD$ values between the groups. Comparisons of mineral loss ($\Delta Z$) and lesion depth (LD) before and after storage/treatment were performed by paired $t$-test (including a Bonferroni correction). All tests were performed at a 5% level of significance.

Manufacturers of the materials mentioned above are named in the respective papers.
4. PRESENTATION OF ORIGINAL MANUSCRIPTS


5. DISCUSSION

Due to their general availability, large size and easy manipulation, bovine teeth were used in the present *in vitro* studies. Bovine specimens are considered suitable to replace human dental tissue for evaluation of demineralization and remineralization effects because of the quite similar behavior during these chemical processes (Arends *et al.*, 1989; Arends *et al.*, 1990; Hara *et al.*, 2003; Kielbassa *et al.*, 2006b; Lynch, 2006). Moreover, since bovine teeth have a more uniform chemical composition, lower variations in the experimental response should be expected (Mellberg, 1992).

The subsurface type of lesion with a well-mineralized surface layer is one of the specific enamel and dentin characteristics that could be clinically observed in initial caries lesions (Arends and Christoffersen, 1986; Schüpbach *et al.*, 1989). Therefore, specimens were demineralized to create subsurface lesions with mineral profiles similar to the typical natural initial caries lesions. After demineralization, specimens were stored for 5 weeks in different solutions (control solutions or saliva substitutes). This period can be considered an intensive contact that is not predictable under clinical conditions. However, saliva substitutes are administered *ad libitum* and, generally, a maximum daily dose is not recommended to patients with hyposalivation. Thus, similar observations might be expected after long periods *in vivo*.

The effects of Saliva natura (polysaccharide-based solution) on dental hard tissues has been evaluated in previous *in vitro* studies (Tschoppe *et al.*, 2007; Tschoppe *et al.*, 2009b). This commercially available artificial saliva (medac, Hamburg, Germany) revealed neutral effects on enamel, but demineralizing effects on dentin. Since no free calcium could be measured in this solution (S$_{OCP} = 0.03$ and S$_{DCPD} = 0.01$), the original product was experimentally modified in order to achieve remineralizing properties on dental hard tissues. Calcium and phosphates were added (ratio 1:1.6, respectively) to this artificial saliva to obtain supersaturated solutions with respect to octacalcium phosphate (S$_{OCP} = 1.0$, 2.0 and 3.0). Additionally, a buffer system (K$_2$HPO$_4$/KH$_2$PO$_4$; ratio 1:2) was incorporated to increase the pH of the modified solutions to 6.0. The experimental Saliva natura solution with S$_{OCP}$ of 2.0 and S$_{DCPD}$ of 1.4 clearly showed the highest remineralization capacity of dentin subsurface lesions in previous experiments (Tschoppe *et al.*, 2009a).
In the first of the present studies, the original product Saliva natura and the experimentally modified solutions (different saturations with respect to OCP) were used in combination with a highly concentrated fluoride toothpaste. After 2 weeks, Saliva natura in combination with the fluoride toothpaste revealed a neutral behavior on dentin subsurface lesions, although a demineralizing effect was verified when specimens were stored in this solution only (Tschoppe et al., 2009a). The additional use of the highly concentrated fluoride toothpaste might have been associated with the demineralization inhibition. However, further demineralization of the dentin specimens was observed after long storage periods (5 weeks) in this artificial saliva. The modified solutions with SOCP of 2.0 and 3.0 demonstrated significantly higher mineral gain compared to the solution with SOCP of 1.0 and to the original product. Although no significant differences in mineral gain could be observed between the solutions with SOCP of 2.0 and 3.0, an increased mineral deposition was detected on specimens stored in the modified solution with SOCP of 3.0. These results suggested that the use of highly concentrated fluorides in combination with saliva substitutes containing calcium and phosphates should be favorable in particular for patients suffering from hyposalivation.

To confirm the benefits of fluoride application in combination with saliva substitutes on dentin remineralization, specimens with subsurface lesions were stored in two different saliva substitutes and treated with Elmex sensitive mouthrinse (250 μg F/g; 10 min; GABA Lörrach, Germany) and/or Duraphat toothpaste (5,000 μg F/g; Colgate, Hamburg, Germany). Non-carbonated mineral water was used as control solution, since moistening of the mouth with water is a simple and inexpensive technique frequently used by many patients with hyposalivation to alleviate their oral symptoms. The mineral water showed a neutral effect that was expected because of the pH value (7.0) and the low saturation of this solution with respect to OCP and DCPD (SOCP = 0.7 and SDCPD = 0.2). The commercially available saliva substitute Glandosane (cell pharm, Hannover, Germany) demonstrated a remarkably demineralizing effect on dentin. Similar effects were observed previously (Smith et al., 2001; Meyer-Lueckel et al., 2002). Glandosane is a carboxymethylcellulose(CMC)-based solution, with a pH value (5.2) lower than the critical value assumed for dentin demineralization (6.0 - 6.5). The pH value, the unspecified amount of titrable acids (sorbic acid and hydrochloric acid), and, consequently, the low saturation with respect to OCP and DCPD (SOCP = 0.3 and SDCPD
= 0.2) could explain the progressive mineral loss induced by Glandosane. In contrast, specimens stored in modified Saliva natura revealed a considerable mineral gain and lesion depth reduction. After 5 weeks, the mineral gain induced by modified Saliva natura was higher in comparison to the other solutions and to the baseline values after demineralization. These results could be explained by the supersaturation of this saliva substitute (SOCP = 2.0 and SDCPD = 1.2).

All fluoride treatments were able to prevent further demineralization of specimens stored in Glandosane. The fluoride treatments with mouthrinse and/or toothpaste should have resulted in a distinct calcium fluoride-like layer on the specimens’ surfaces (Christoffersen et al., 1988; Ogaard, 2001), which should have been dissolved over time by the potentially demineralizing effects of Glandosane. These precipitates on the specimens’ surface might have acted as a fluoride reservoir, thus hampering the demineralization caused by Glandosane (Ogaard, 2001). Modified Saliva natura revealed a remineralizing effect, and the additional use of only one fluoride product did not enhance remineralization of specimens stored in this saliva substitute. However, mineral deposition on the lesion body and surface layer was significantly increased after storage in modified Saliva natura and treatment with both fluoride products. Recently, a more pronounced remineralization of subsurface lesions was observed with an acidic solution compared to a neutral remineralizing one (Yamazaki and Margolis, 2008). This could explain the absence of mineral gain with the use of the neutral highly concentrated toothpaste compared to the combination of toothpaste/acidic mouthrinse, and this would be in accordance with a previous paper reporting a significant reversal of primary root caries lesions after use of a toothpaste/fluoride mouthrinse (Petersson et al., 2007).

It has been suggested that root dentin requires a significantly higher level of fluoride uptake and retention to enhance remineralization (Featherstone, 1999; ten Cate, 1999; ten Cate, 2001). Indeed, previous in vitro studies have demonstrated that higher fluoride concentrations are necessary for caries inhibition in dentin compared to enamel (Herkstroter et al., 1991; Mukai et al., 2001). Therefore, the same protocol used to evaluate the effects of fluoride products in combination with saliva substitutes on demineralized dentin was applied for enamel specimens.
After 2 and 5 weeks, specimens stored in Glandosane demonstrated an accentuated demineralization. This negative effect could be completely inhibited by daily application of fluoride, without significant differences between the fluoride treatments. Although these results were similar to the results obtained with dentin specimens, the observed demineralizing effect of Glandosane was more pronounced on dentin. The difference in mineral loss ($\Delta\Delta Z$) after 5 weeks was -2932 for enamel and -3795 for dentin. On the other hand, modified Saliva natura enabled significant remineralization of enamel lesions. However, additional effects on remineralization could not be verified with the use of fluoride products. For dentin specimens, additional remineralization could be observed after treatment with Elmex Sensitive mouthrinse and Duraphat toothpaste. The difference in mineral loss ($\Delta\Delta Z$) observed for enamel specimens stored only in modified Saliva natura was +1570, which was close to the value verified for dentin specimens after storage in the same saliva substitute and treatment with both fluoride products (+1423). Moreover, the mean graphs of mineral loss ($\Delta Z$) demonstrate that topical application of fluoride on the enamel specimens stored in modified Saliva natura resulted in a more mineralized lesion surface. It may be speculated that precipitates of calcium fluoride or calcium fluoride-like material on the surface of the specimens treated additionally with the fluorides might have blocked the diffusion pathways into the enamel subsurface area (Ogaard, 2001).

The number of older adults who have retained their natural teeth is increasing (Nicolau et al., 2000); thus, clinicians now face a new caries challenge in older dentate patients (Shay, 1997; Griffin et al., 2004; Saunders and Meyerowitz, 2005). The physiological (or pathological) gingival recession usually observed in these patients will increase the risk of root surface caries development (Joshi et al., 1993; Shay, 1997). Moreover, several determinant factors may influence caries risk at the individual level, such as oral hygiene, frequency of fermentable carbohydrates consumption, and salivary aspects (e.g., flow, remineralizing potential, buffer capacity, cleaning properties, and defensive factors) (Curzon and Preston, 2004). More than half of the elderly who are dentate are affected with either coronal or root caries, and caries is the primary cause of tooth loss in this population (Saunders and Meyerowitz, 2005). Although fluoride is considered effective in reducing caries levels, it seems to be insufficient to overcome high caries challenge in many individuals such as persons with reduced salivary function. In these patients, new approaches should be found to enhance the remineralization process.
(Featherstone, 2009). Therefore, the general aim of this study was to evaluate the effects of different saliva substitutes in combination with or without fluoride products on dentin and enamel subsurface lesions remineralization.

Data from the present studies highlight that any choice of saliva substitute should be made carefully to avoid introducing substances that might have potentially demineralizing effects on dental hard tissues. Topical fluoride application in combination with a strict oral hygiene has become a standard practice to prevent caries lesions in patients with hyposalivation. However, the compliance of patients with the use of fluoride products after radiation therapy of the head and neck region is generally thought to be poor (Epstein et al., 1996). For this reason, the use of a remineralizing artificial saliva such as modified Saliva natura should be a promising approach for dentate patients suffering from hyposalivation to manage both dental caries as well as hyposalivation.
6. CONCLUSIONS

Within the limitations of the present \textit{in vitro} studies, it could be concluded that Glandosane is a demineralizing saliva substitute that should only be used in combination with fluoride products by dentate patients suffering from hyposalivation.

On the other hand, modified Saliva natura supersaturated with respect to calcium phosphates seems to enable remineralization of dentin and enamel subsurface lesions. For demineralized dentin, an additional mineral gain could be observed with daily application of Elmex Sensitive mouthrinse and Duraphat toothpaste. The use of modified Saliva natura should be strongly recommended for dentate patients with salivary hypofunction since lack of calcium and phosphate ions which are present in natural saliva is considered the limiting factor of remineralization of carious lesions, even in the presence of high fluoride concentrations.

In general, the results of these \textit{in vitro} studies should be extrapolated with caution to the clinical conditions. Therefore, well designed \textit{in situ} and \textit{in vivo} studies should be performed to confirm the potential benefits of the use of modified Saliva natura in combination or not with fluoride products by patients with hyposalivation.
7. ABSTRACT

Statement of problem: Hyposalivation is the most common side effect of radiation therapy in the head and neck areas, but the reduction of salivary flow rates can also be associated with prolonged use of certain drugs, or some diseases (Sjögren's syndrome, diabetes mellitus). Moistening of the oral mucosa with saliva substitutes is the widely prescribed palliative treatment to alleviate oral complaints in patients with hyposalivation. However, some commercially available products have been shown to demineralize dental hard tissues. Objectives: Therefore, the general purpose of the present in vitro studies was to evaluate the effects of saliva substitutes in combination with or without fluoride products on the remineralization of dentin and enamel subsurface lesions. Material and Methods: Remineralization of predemineralized specimens (subsurface lesions) was microradiographically evaluated after application of different protocols. These protocols included storage of specimens in control solutions or saliva substitutes (37 °C) for 5 weeks. During this period, specimens were treated with or without fluoride products. Results: The addition of calcium and phosphate ions to the commercially available saliva substitute Saliva natura (medac) enabled dentin and enamel remineralization. In contrast, storage in Glandosane (cell pharm) resulted in pronounced demineralizing effects on dental hard tissues. This detrimental effect was reduced or inhibited by daily fluoride applications. The additional treatment with fluoride products enhanced the remineralizing effect of modified Saliva natura only for dentin specimens. Conclusions: Based on the results of these in vitro studies, it could be concluded that Glandosane is a demineralizing saliva substitute that should only be used in combination with frequently applied fluorides in dentate patients. Modified Saliva natura enables remineralization of enamel and dentin subsurface lesions.

Keywords: bovine; dentin; enamel; demineralization; fluoride; hyposalivation; in vitro; microradiography; remineralization; saliva substitute

Clinical Significance: Data from the present studies demonstrated that the commercially available saliva substitute Glandosane might have demineralizing effects on dental tissues if not used in combination with fluoride products. Saliva natura supersaturated with respect to calcium phosphates should be a particularly advantageous artificial saliva for dentate patients suffering from hyposalivation.
8. ZUSAMMENFASSUNG


Schlagwörter: Dentin; Demineralisation; Fluoride; Hyposalivation; in vitro; Mikroradiografie; Remineralisation; Speichelersatzmittel

9. REFERENCES


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I can confidently say that the experience acquired during the development of this thesis will have a pronounced influence on my career.
11. CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.
12. EIDESSTATTLICHE ERKLÄRUNG

„Ich, Daniela Leal Zandim, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema “The Effect of Daily Fluoride Applications in Combination with Saliva Substitutes on Remineralization of Bovine Dentin and Enamel Subsurface Lesions” selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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